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THE ROLE OF INSULIN AND GROWTH
HORMONE IN THE REGULATION OF HEPATIC
STEROID METABOLISM

A thesis submitted to the
University of Glasgow
in candidature for the degree of
Doctor of Philosophy
in the
Faculty of Medicine
by
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SUMMARY

The hepatic monooxygenase system catalyses the oxidative metabolism of both endogenous steroids and xenobiotics, and consists of three main components : cytochrome P-450, NADPH cytochrome P-450 reductase and lipid (reviewed by Skett 1987). Multiple pathways exist for cytochrome P-450-catalysed biotransformation in the liver, and these pathways are modulated by a large number of environmental and hormonal factors (reviewed by Mathis et al 1988). In particular, growth hormone plays a central role in the regulation of hepatic drug and steroid metabolism (Waxman 1988).

Sex differences in hepatic biotransformations have been widely documented in the rat. Neonatal androgenic imprinting of cytochrome P-450 is mediated via the hypothalamo-pituitary-liver axis (Skett and Gustafsson 1979) by modulations of the GH secretory pattern (Edén 1979). Thus pulsatile GH secretion in male rats results in the predominance of oxidative drug and steroid metabolism, whilst continuous GH secretion in the female results in higher reductase activity (Mode et al 1982). Alterations of GH secretion towards a more continuous pattern have been correlated with the feminisation of hepatic steroid metabolism (Mode et al 1981). In addition, insulin has been documented to exert a "masculinising" influence on this system (Skett 1986).

The majority of studies carried out in this field have used in vivo animal models (Kramer et al 1979), isolated perfused livers (Schalch et al 1979), and liver microsomes prepared from pretreated rats (Gustafsson and Stenberg 1974). However each of these models suffer from a number of disadvantages, and their use is now becoming limited and is being largely replaced by cultured hepatocytes. This is an important technique in that it is a relatively physiological preparation, and allows the study of the effect of individual hormones in this system without the interactions caused by administration of exogenous hormones to the intact animal.

The primary aim of this project was to determine whether or not GH produced its characteristic feminising effect on steroid metabolism in isolated hepatocytes, and to correlate the results obtained with the situation in vivo.

Incubation of hepatocytes with GH caused no significant changes in either hepatic steroid metabolism or intracellular cyclic AMP levels. Previous reports have suggested that the lack of effects of GH in vitro may be due to the absence of additional hormonal factors that are involved in the intact animal (Colby 1980). We therefore initiated studies to determine the interactions of GH with insulin, dexamethasone and thyroxine, each of which is known to influence hepatic drug and steroid metabolism (Skett 1987). These effects are substantiated by studies in adrenalectomised, thyroidectomised or diabetic animals,

where removal of a particular hormone markedly altered drug metabolism (Kato and Gillette 1965).

Insulin stimulated steroid metabolism in a non-specific manner, as has been reported previously (Hussin and Skett 1987), an effect that was potently antagonised by GH. Pyerin and coworkers, in a series of articles, have documented the phosphorylation of cytochrome P-450 by both cyclic AMP-dependent and independent protein kinases concomitant with the denaturation / inactivation of cytochrome P-450 (Pyerin et al 1987). A phosphorylation mechanism appears to be involved in the action of insulin to stimulate steroid metabolism, and so altered phosphorylation of the cytochrome P-450 protein may constitute this effect. The interaction observed with GH may also involve a phosphorylation mechanism, as has been reported elsewhere (Yamada et al 1987).

Dexamethasone stimulated steroid metabolism in a non-specific manner, and this effect was again antagonised by GH. The effect of dexamethasone over short-term incubations may represent a glucocorticoid effect, while the effects of long term incubation probably represents induction of cytochrome P-450_{PCN} (gene IIIA1) (Schuetz et al 1984). The interaction with GH may involve its action to suppress cytochrome P-450 expression (Yamazoe et al 1987, Waxman et al 1988a).

Incubation with thyroxine exhibited dose-related and sex-dependent effects in the short incubation studies, while

no significant effects were observed over the longer incubations, except at 72h of incubation. Further incubation with GH decreased steroid metabolism markedly in the former, but enzyme activity was maintained at control levels in cultured hepatocytes over 72hr of incubation.

GH modified the effects of insulin, dexamethasone and thyroxine in a non-sex-specific manner. Incubation of hepatocytes with GH in the presence of both dexamethasone and thyroxine, however, resulted in an increase in female-specific activities together with a decrease in male-specific activities, which is characteristic of the feminisation of steroid metabolism. The role of the permissive hormones, dexamethasone and thyroxine (Malbon et al 1988), is unclear at this stage. It is possible that inclusion of these hormones simply serves to mimic the hormonal environment in vivo, and that GH interacts with both dexamethasone and thyroxine in producing the feminising effect. A synergistic effect of cortisol and thyroxine on the stimulation of cytochrome P-450_{15β} (gene IIC12) by GH has recently been documented (Mode et al 1989). It has also been suggested that both glucocorticoids and thyroxine are required for the maintenance of GH receptors, and therefore GH responsiveness, in cultured hepatocytes (Christoffersen et al 1984, Duran-Garcia et al 1987).

Studies with the putative insulin mediator extract isolated from hepatocytes showed that the effects of insulin to stimulate steroid metabolism may be mediated by this

inositol phosphate glycan (IPG) (Saltiel 1987). Thus the insulin mediator stimulated steroid metabolism in a dose-and concentration-dependent manner. In addition, this effect was maximal at physiologically relevant concentrations, as was seen with insulin. GH antagonised the stimulatory effect of the insulin mediator, indicating that the level of interaction between the two hormones was distal to the point of insulin mediator generation.

Chromatography of the AG1x8-purified acid-hydrolysed insulin mediator indicated the presence of carbohydrates, as has been documented before (Larner et al 1988, Saltiel et al 1987), although a more sensitive technique is required to fully distinguish between the components on chromatography.

Effect of GH on insulin mediator-stimulated steroid metabolism in the presence of GH. GH (10⁻⁶ M) inhibited the insulin mediator-stimulated steroid metabolism in a dose-dependent manner. The effect of GH on insulin mediator-stimulated steroid metabolism was not observed in the absence of insulin mediator. The effect of GH on insulin mediator-stimulated steroid metabolism was not observed in the absence of insulin mediator. The effect of GH on insulin mediator-stimulated steroid metabolism was not observed in the absence of insulin mediator.

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ABBREVIATIONS

ACTH	: Adrenocorticotrophin
ADP	: Adenosine-5'-diphosphate
cyclic AMP	: Cyclic adenosine-3',5'-monophosphate
ATP	: Adenosine-5'-triphosphate
BSA	: Bovine serum albumin
Cys	: Cysteine
DAG	: Diacylglycerol
DEX	: Dexamethasone
DMSO	: Dimethylsulphoxide
DNA	: Deoxyribonucleic acid
EGF	: Epidermal growth factor
FAD	: Flavin adenine dinucleotide
FMN	: Flavin mononucleotide
FSH	: Follicle stimulating hormone
G-protein	: Guanine nucleotide regulatory protein
G _i	: Inhibitory guanine nucleotide regulatory protein
G _{i ns}	: Guanine nucleotide regulatory protein specific for insulin
G _s	: Stimulatory guanine nucleotide regulatory protein
GH	: Growth hormone
GHRH	: Growth hormone-releasing hormone
Gly	: Glycine
cyclic GMP	: Cyclic guanosine-3',5'-monophosphate
IBMX	: Isobutylmethylxanthine

IGF	: Insulin-like growth factor
IM	: Insulin mediator
INS	: Insulin
IP	: Inositol phosphate
IPG	: Inositol phosphate glycan
LH	: Luteinising hormone
NADH	: Reduced nicotinamide adenine dinucleotide
NADPH	: Reduced nicotinamide adenine dinucleotide phosphate
PDH	: Pyruvate dehydrogenase
PK A	: Cyclic AMP-dependent protein kinase
PK C	: Calmodulin-phospholipid-dependent protein kinase
PI	: Phosphatidylinositol
PL _C	: Phospholipase C
PMA	: 4 β -phorbol-12 β -myristate-13 α -acetate
PRL	: Prolactin
mRNA	: Messenger ribonucleic acid
Ser	: Serine
SD	: Standard deviation
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM-C	: Somatomedin C
STZ	: Streptozotocin
T ₃	: Triiodothyronine
T ₄	: Thyroxine
Tyr	: Tyrosine

INTRODUCTION

INTRODUCTION

1.1 HISTORICAL BACKGROUND : CYTOCHROME P-450

The cytochromes P-450 comprise a superfamily of enzymes that play a central role in the oxidative biotransformation of both endogenous compounds, such as steroids and eicosanoids, and exogenous substances (e.g. xenobiotics).

A vast amount of information has accumulated over the past four decades regarding the cytochrome P-450 system : Mueller and Miller first reported in 1949 that certain carcinogens were metabolised by liver microsomes supplemented with NADPH. Brodie et al (1955) subsequently reported that these enzymes, mediating biotransformation of compounds, were located in the endoplasmic reticulum of the liver parenchymal cells. Mason (1957) characterised the process of mixed-function oxidation, although the relevance of this finding was not fully realised at that time. The cytochrome P-450 pigment was discovered by Garfinkel (1958) and Klingenberg (1958), although once again the significance of this protein was not recognised. Omura and Sato (1964) first demonstrated this cytochrome P-450 protein as being the terminal oxidase in the adrenal mixed function oxidase system using the carbon monoxide spectrum. In addition, their observation of intense bands in the Soret region of the ethyl isocyanide difference spectrum of reduced microsomes revealed the haemoprotein nature of the pigment. They named it cytochrome P-450, and further showed that

detergent solubilised the pigment and converted it to a carbon monoxide form with maximum absorption at 420nm. Cooper et al (1965) subsequently assigned a physiological role to the cytochrome P-450 system.

The role of the protease-solubilised microsomal NADPH cytochrome c reductase described by Horecker (1950) was then established as being part of the NADPH-cytochrome P-450 reductase protein. Strittmatter and Velick (1956) documented the microsomal origin of this protein. Lu and Coon (1968) solubilised rabbit liver microsomes and separated fractions containing cytochrome P-450, NADPH-cytochrome P-450 reductase and phospholipid, and showed catalytic activity upon combining these fractions in a reconstituted system.

The diversity of substrates metabolised by the cytochrome P-450 system led to the suggestion that isoenzymes of cytochrome P-450 may be present (Alvares et al 1967). Cytochrome P-450 has subsequently been isolated, purified and characterised by a number of groups (Ryan et al 1975, Guengerich 1977) and the idea of a distinct family of isoenzymes with unique yet overlapping substrate specificities was proposed by Lu and West (1980). Since then primary sequences of some cytochromes P-450 and their genes have been determined (Black and Coon, 1986) as well as the correlation of different isoenzymes with specific reactions (Waxman 1984, Morgan et al 1985a).

Many studies have established the importance of gonadal hormones in the regulation of hepatic drug and steroid metabolism in rats (Yates et al 1958, Conney 1967, Gillette et al 1972). Corticosteroid metabolism in rats was shown to

be enhanced by oestradiol and diminished by testosterone administration, resulting in a higher rate of corticosteroid metabolism in females than males (Forchielli and Dorfman, 1956). The existence of sex differences in hepatic metabolism was first reported by Nicholas and Barron (1932) and Holck et al (1937) who showed that the barbiturate dose required to anaesthetise male animals was twice that in females, indicating a sex difference in hexobarbital metabolism. Sex differences in the metabolism of gonadal steroids have also been reported (Yates et al 1958, Conney 1967).

Several groups have isolated male-specific and female-specific cytochrome P-450 forms (Kamataki et al 1981). Colby et al (1973, 1974) have indicated the requirement of the pituitary gland in mediating the effects of oestrogens and androgens on hepatic metabolism, as well as for the maintenance of sex differences in hepatic metabolism (Wilson 1968, Gustafsson and Stenberg 1974a, Deneff 1974). Gustafssons' group subsequently showed the existence of a pituitary "feminising factor" in the regulation of steroid metabolism (Gustafsson and Stenberg 1974) and this factor has been identified as growth hormone (Mode et al 1983).

Kato and co-workers have also indicated a role of the gonadal hormones (Kato et al 1969), thyroid hormones (Kato and Takahashi 1968), adrenal steroids (Kato and Gillette 1965) and insulin (Kato and Gillette 1965) in the regulation of this system, a view that has been reinforced by subsequent reports (Colby et al 1974).

1.2 COMPONENTS OF THE CYTOCHROME P-450 SYSTEM

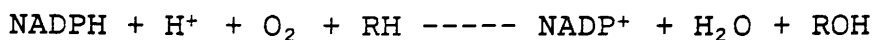
The metabolism of drugs and steroids can be divided into two categories. Phase I metabolism primarily catalyses the oxidation, reduction, hydrolysis and hydration of xenobiotics. Oxidative metabolism can then be further categorised into the mixed-function oxidases and cytochrome P-450-independent reactions (such as alcohol dehydrogenase). Thus Phase I metabolism ("functionalisation") converts the xenobiotic to a chemically reactive form, thereby making it available for Phase II metabolism, whereas Phase II metabolism is a conjugation reaction which enhances the elimination of the xenobiotic from the body.

The enzymes responsible for drug metabolism are known to be present and functional in the lung, kidney, gut, adrenal cortex, skin, brain, aorta and placenta, although the liver is the major site for these enzymes. The phase I mixed-function oxidases are exclusively present embedded in the endoplasmic reticulum, while the phase II enzymes (except for UDP-glucuronyltransferase) are primarily resident in the cell cytoplasm. The components and mechanisms involved in the monooxygenase system are discussed below.

Lu and Coon (1968) first demonstrated catalytic activity of solubilised components of the cytochrome P-450 system in a reconstituted system. Further, they showed that two other components, NADPH cytochrome P-450 reductase and lipid were important to achieve maximal activity in a reconstituted system, and assigned a role to each of the

components. Thus, cytochrome P-450 was responsible for binding to molecular oxygen and substrate, and upon reduction, released one atom of oxygen as water and transferred the second atom to the substrate; the NADPH cytochrome P-450 reductase transferred reducing electrons from NADPH to cytochrome P-450; and the lipid component appeared to function as a matrix to allow effective interaction between the reductase and cytochrome P-450.

Thus the cytochrome P-450-catalysed mixed function oxidase reaction is :



An additional cytochrome P-450-linked monooxygenase system, whose role is probably to synergise with NADPH-dependent metabolism involves NADH cytochrome b_5 reductase, cytochrome b_5 and cytochrome P-450.

1.2.1.1 CYTOCHROME P-450

Cytochrome P-450 is a haemoprotein which contains iron protoporphyrin IX as the prosthetic group (Maines and Anders 1973). Four of the six ligands of the iron of cytochrome P-450 are co-ordinated with pyrrole nitrogens. The fifth ligand is a sulphur atom (Waterman and Mason 1972), probably associated with a cysteine residue that has been identified close to the haem moiety (Dus et al 1976). The exact nature of the sixth ligand has not been determined, although both a nitrogen histidyl imidazole group (Dus et al 1970) and water (Griffin and Peterson 1973) have been suggested as suitable candidates.

Omura and Sato (1964) first showed that the cytochrome P-450 haemoprotein, in its reduced state, readily combines with carbon monoxide to yield an absorption maximum at 450nm. Cytochrome P-450 exists in high- and low-spin forms both in the absence and presence of substrates (Sliger et al 1976). At any given time, the iron of a single molecule of cytochrome P-450 is either in the low-spin state ($s=1/2$ ie one unpaired d-electron) or high-spin state ($s=5/2$ ie five unpaired d-electrons). The time spent in each state determines the equilibrium and this is established by the conformation of the protein associated with the haem (Hall 1987).

Perturbations of the protein may alter the spin state equilibrium. Thus the hydrophobic Type I substrates (eg hexobarbitone, benzphetamine, endogenous steroids), which bind to low-spin cytochrome P-450, perturb the protein by combining with the substrate binding site located on a hydrophobic region of the protein near the sixth ligand of the iron. When the substrate combines with its binding site, the weak sixth ligand is displaced and the 6-coordinated iron (low-spin, 418nm Soret minimum) shifts to the 5-coordinated iron (high-spin, 393nm Soret maximum) (Kumaki and Nebert 1978). This results in a shift of the equilibrium towards a high-spin state. This change in configuration from low-spin to high-spin is characterised as a Type I spectral change, and exhibits an absorption maxima at 390nm and a minima at 420nm.

Type II compounds, such as aniline, are mainly amines and bind to the haem iron of cytochrome P-450 (Kumaki and

Nebert 1978) to form a strong ligand that maintains the iron in the low-spin 6-coordinated state. This causes a shift in the equilibrium towards the low-spin state.

1.2.1.2 NADPH CYTOCHROME P-450 REDUCTASE

Horecker (1950) first characterised this NADPH-linked reductase activity. The reductase contains one molecule each of FMN and FAD as the prosthetic group (Iyanagi and Mason 1973). The role of NADPH cytochrome P-450 reductase appears to be the transfer of reducing electrons from NADPH to cytochrome P-450 (Lu and Coon 1968). The FAD acts as the acceptor flavin from NADPH while FMN appears to be the donating flavin to cytochrome P-450 during electron transfer (Vermilion and Coon 1978, Iyanagi et al 1977).

Five functional domains in NADPH cytochrome P-450 reductase have been identified. These include a hydrophobic amino-terminal membrane-binding domain, which is also required for the reduction of cytochrome P-450 (Black et al 1979, Gum and Strobel 1981) and hydrophilic regions involved in FMN, FAD, NADPH and cytochrome P-450 binding (Porter and Kasper 1986, Nisimoto 1986). Further, residues Tyr-140 and Tyr-178 are involved in binding FMN to NADPH cytochrome P-450 reductase (Shen et al 1989). Carboxyl groups on the NADPH cytochrome P-450 reductase also appear to be involved in the interaction with cytochrome P-450 during electron transfer and in the rate of hydroxylation of substrates, therefore suggesting that electrostatic interactions play an important role in the binding and electron transfer steps

between cytochrome P-450 and the reductase (Nadler and Strobel 1988).

The stoichiometry of this reaction shows that approximately 20-30 molecules of cytochrome P-450 are present to one NADPH cytochrome P-450 reductase (Shiraki and Guengerich). This may therefore be the rate-determining step in this reaction (Miwa et al 1978, Dutton et al 1987).

1.2.1.3 LIPID

Lipid, in the form of phosphatidylcholine, is thought to provide a matrix for effective interaction between cytochrome P-450 and NADPH cytochrome P-450 reductase (Lu and Coon 1974). Further, sex differences in microsomal lipid composition are known to exist, and are reflected in the drug-metabolising activities in reconstituted systems (Barr and Skett 1984). In addition, Meftah and Skett (1987a & b) have more recently shown that the lipid component is an important requirement for the maintenance of the sex differences observed in hepatic drug metabolism.

1.2.1.4 CYTOCHROME b_5 REDUCTASE

The addition of NADH to an oxidising system was reported to have a synergistic effect with NADPH (Conney et al 1957). Strittmatter and Velick (1957) isolated an NADH-specific reductase with cytochrome b_5 as the intermediate transfer component. Cytochrome b_5 reductase transfers electrons from NADH to cytochrome P-450 at the

level of the second electron only (Imai and Sato 1977). Reduction of cytochrome P-450 by cytochrome b₅ instead of NADPH-cytochrome P-450 reductase (first electron transfer) was reported to be inhibitory on hepatic drug metabolism (Golly et al 1988).

1.2.2 BIOCHEMICAL MECHANISMS

Figure 1 illustrates the sequence of events in a typical cytochrome P-450-catalysed drug-oxidation. The catalytic cycle of cytochrome P-450 (reviewed by White and Coon 1980, Hall 1987) consists of two parts, oxygen activation and oxygen insertion, and proceeds from steps 1 to 7 :

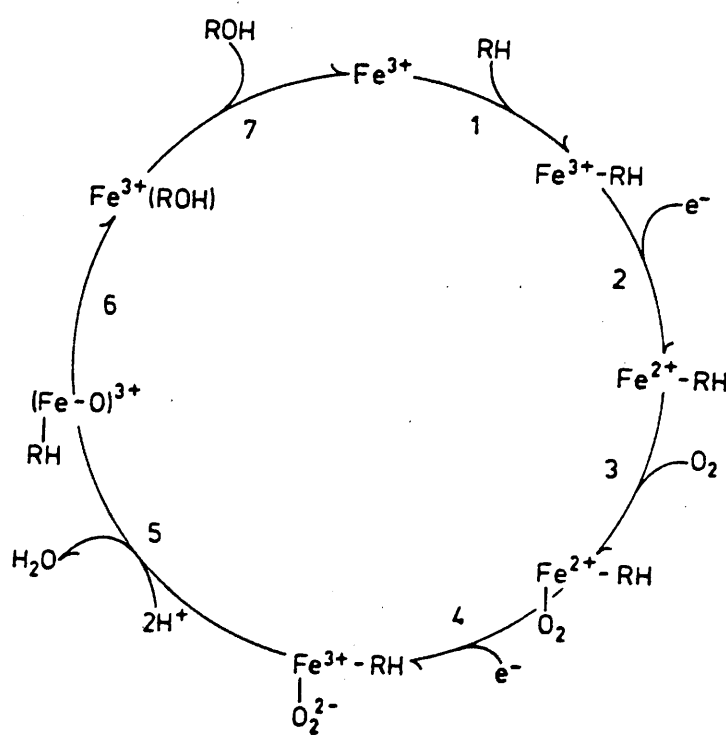
Step 1 :

The oxidised or ferric form of cytochrome P-450 binds to the substrate. Three classes of substrate have been proposed (Schenkman et al 1967), Type I, Type II and reverse Type I (or modified Type II). Type I substrates are hydrophobic in nature and therefore bind to the hydrophobic moiety of cytochrome P-450 causing a type I spectral change (absorption maxima at 390nm and minima at 420nm). Type II substrates are mainly amines and interact with the haem prosthetic group, and are characterised by absorption maxima and minima at 325nm and 425-435nm respectively. The third class, reverse Type I substrates characteristically display absorption minima at 390nm and maxima at 420nm (Hall 1987).

Step 2:

The cytochrome P-450 complex in the ferric oxidation

FIGURE 1 : CATALYTIC CYCLE OF CYTOCHROME P-450.



state undergoes a one-electron reduction to the ferrous state (Peters et al 1965). The electron originates from NADPH and is transferred to the cytochrome P-450 complex by cytochrome P-450 reductase.

Step 3:

The reduced cytochrome P-450-substrate complex (ferrous form) reacts with molecular oxygen to form a tertiary P-450-O₂-substrate complex (oxyferrous complex). Alternatively, the complex can bind carbon monoxide to produce an absorption maximum at about 450nm (Omura and Sato 1964).

Step 4:

A second one-electron insertion takes place which re-oxidises the iron to its ferric form and the oxygen molecule is activated (White and Coon 1980). The source of this second electron can be NADPH cytochrome P-450 reductase or NADH cytochrome b₅ reductase (Imai and Sato 1977). The extent of involvement of cytochrome b₅ depends upon the form of cytochrome P-450 involved (Imai 1981).

Step 5:

The two oxygen atoms separate at this stage and water is released, resulting in the formation of an oxene intermediate which subsequently oxidises the substrate. This oxene intermediate is atomic oxygen bound to the ferric haemoprotein (Feⁱⁱⁱ-O₂ complex) and is a potent oxidising agent since it is highly electron deficient. Very little else is known about this step.

Step 6:

The oxygen atom of the oxene intermediate is

transferred to the substrate to form the hydroxylated form.

Step 7:

The hydroxylated product is subsequently released from the enzyme complex and the iron haemoprotein returns to the ferric state.

Monooxygenase reactions take place on or near the outer surface of the membrane. Membrane topology theories of cytochrome P-450 are an extension of the studies carried out using bacterial cytochrome P-450_{c_am} (gene CIA1). In addition, the location and sequence of α -helical regions in mammalian cytochrome P-450 have been predicted from their homology with cytochrome P-450_{c_am} (Haniu et al 1986, Edwards et al 1989). Cytochrome P-450 is thought to be bound to the endoplasmic reticulum by one or two transmembrane hydrophobic segments located at the amino-terminus of the polypeptide chain (Nelson and Strobel 1988, Brown and Black 1989). The remainder of the protein, comprising the haem moiety, is thought to be exposed to the cytosolic side of the membrane. The catalytic domain of cytochrome P-450 must therefore be orientated in a manner that is complementary to the arrangement of reductase and substrate, so as to allow adequate electron transfer. The hydrophobic portion of NADPH cytochrome P-450 reductase, which accounts for about 10% of the molecule, is embedded in the membrane with its catalytic site in juxtaposition with the catalytic site of cytochrome P-450 (section 1.2.1.2). Similar hydrophobic regions of NADH cytochrome b₅ reductase and cytochrome b₅ also provide the opportunity for their interaction at the catalytic site of

cytochrome P-450 (Kensil and Strittmatter 1986, Arinç et al 1987). These cytochrome P-450 molecules are believed to congregate around a single molecule of the reductase such that the reductase can react with one and then with another of the cytochrome P-450 molecules (French et al 1980).

1.2.3 SYNTHESIS AND DEGRADATION OF CYTOCHROME P-450

The cytochrome P-450 isoenzymes consist of both a haem prosthetic group and a protein component. The haem is synthesised by the haem biosynthetic pathway (Granick and Urata 1963), and the protein component by the normal method of protein synthesis, that is, transcription and translation. The protein component of cytochrome P-450 is responsible for the presence of isoenzymes, although marked amino acid sequence homologies have been reported within gene families (Black and Coon 1986). The cytochrome P-450 holoenzyme is formed upon incorporation of the haem into the apoprotein of cytochrome P-450 (Bornheim et al 1987). Inhibitors of haem biosynthesis blocked the induction of cytochrome P-450 and its transcription by 3-methylcholanthrene, thus implying that haem acts as a positive regulator of cytochrome P-450 gene transcription (Bhat and Padmanaban 1988).

Cytochrome P-450 is degraded by the haem oxygenase system (Maines et al 1986). This system consists of one of the two isoforms of haem oxygenase, and NADPH cytochrome P-450 reductase, and utilises NADPH (or NADH) and molecular oxygen to catalyse the cleavage of the haem molecule at the

α -meso bridge to form the biliverdin IX α -isomer (Kutty et al 1988). In turn, biliverdin is converted into bilirubin in the course of catalytic activity of isomer-specific biliverdin reductase.

1.3 REGULATION OF CYTOCHROME P-450-DEPENDENT STEROID METABOLISM

1.3.1 ROLE OF GONADAL HORMONES

The rat hepatic drug- and steroid-metabolising system exhibits sex-related differences in metabolism around the onset of puberty (Stenberg 1976). Adult males have higher hydroxylating activity, whereas metabolism is primarily reductive in adult females. In general, androgens increase oxidative metabolism and decrease reductive metabolism, whilst oestrogens exert the opposite effects (Kato et al 1969, Kramer et al 1978, Berg and Gustafsson 1973, Mode and Norstedt 1982).

Steroid hormones are thought to be the physiological correlates of xenobiotics in hepatic P-450 metabolism (Gustafsson and Stenberg 1974a) which is supported by the finding that steroid metabolism proceeds at lower K_m values than for xenobiotic metabolism, indicating that steroids may be the preferred substrates in vivo (Skett 1978).

The significance of sex differences in hepatic steroid metabolism are, as yet, unclear. One possible explanation could be the maintenance of sex hormone concentrations. For example, the high 5α -reductase activity in females rapidly

converts testosterone to 5 α -dihydrotestosterone, whilst oestrogens are readily hydroxylated in males. This would result in the metabolism and excretion of unwanted steroids in the animal.

It is now widely accepted that gonadal hormones play a vital role in the regulation of hepatic drug and steroid metabolism. Firstly, sex-dependent effects in hepatic metabolism are not observed in male and female rats until the onset of puberty (DeMoor and Denef 1968, Stenberg 1976, Berg and Gustafsson 1973). Secondly, castration of male animals at different ages resulted in differences in hepatic steroid metabolism (Berg and Gustafsson 1973, Kato et al 1969).

Yates et al (1958) showed that castration of adult male rats resulted in an increase in 5 α -reductase activity, an effect that was reversed upon treatment with testosterone. In a similar set of experiments, it was shown that castration of adult male rats resulted in an increase in the female-specific 5 α -reductase activity together with a decrease in hydroxylating activity (Einarsson et al 1973, Kato et al 1969, Berg and Gustafsson 1973), and that this "feminisation" of male liver enzymes could be reversed by testosterone treatment. In contrast, ovariectomy had no effects on steroid metabolism (Gustafsson and Stenberg 1974b), although oestrogen administration to male rats resulted in a feminisation of steroid metabolism (Skett 1978). These preliminary results therefore suggested that, although androgens were necessary for maintaining a "male-type" of metabolism, oestrogens were relatively

unimportant for "female-type" activities.

1.3.2 NEONATAL IMPRINTING

Gustafsson and Stenberg (1974b) showed that hepatic steroid metabolism in neonatally castrated males could not be "remasculinised" upon postpubertal treatment with testosterone. Conversely, steroid metabolism in adult female rats could not be masculinised upon postpubertal androgen treatment. The authors therefore proposed the phenomenon of "neonatal imprinting" (Gustafsson and Stenberg 1974c, Gustafsson et al 1975, Gustafsson and Stenberg 1976, Gustafsson et al 1977), that exposure to androgens in the neonatal period was essential for the expression of the adult male type of steroid metabolism (reviewed by Skett and Gustafsson 1979). The absence of neonatal androgens in the female therefore resulted in the expression of female-specific activities (5 α -reductase, 15 β -hydroxylase) and the relative absence of male-specific hydroxylating enzymes.

Einarsson et al (1973) suggested that the enzymes responsible for steroid metabolism could be divided into three categories according to their developmental regulation :

I : This group is best represented by 6 β -hydroxylase activity, which is two to three times higher in male than female rats. This sexual difference was completely abolished both by neonatal and postpubertal testectomy, and stimulated

by testosterone propionate treatment. 6β -hydroxylase activity could also be stimulated in castrated female rats upon testosterone administration, although not to levels in the male. These results therefore suggest that this group of enzymes has a basal activity regulated by nongonadal factors and is reversibly induced by androgen treatment.

II : The second group of enzymes, typified by 16α -hydroxylase activity was generally more active in male than female rats. Only neonatal testectomy completely abolished this sexual difference whereas postpubertal testectomy reduced but did not abolish the sex difference in enzyme activity between male and female animals. Testosterone propionate administration to postpubertally gonadectomised male rats restored 16α -hydroxylase activity. Testosterone treatment of castrated female rats also resulted in an increase in enzyme activity. This group of enzymes is therefore irreversibly imprinted neonatally and reversibly stimulated postpubertally by androgens. 17 -oxosteroid oxidoreductase activity is also regulated in this manner.

5α -reductase activity is about ten fold higher in female than male animals. This sex difference in enzyme activity, similar to that of a Group II enzyme, was neonatally imprinted and partially controlled by androgens postpubertally. 15β -hydroxylase activity has only been detected in females and can also be characterised as a Group II enzyme.

Group II enzymes can be further subdivided into three groups according to their sensitivity to imprinting (Gustafsson and Stenberg 1974b) :

- a) Enzymes imprinted by a low dose of androgen and imprinting is complete within 7 days e.g. 5 α -reductase;
- b) Enzymes imprinted by a higher dose of androgen and imprinting takes 7 days;
- c) Imprinting is not complete by 30 days of age e.g. 16 α -hydroxylase.

III : The third category (Einarsson et al 1973), exemplified by 7 α -hydroxylase activity, is generally more active in female than male rats. Neonatal or postpubertal testectomy had little effects on enzyme activity, indicating that this group of enzymes is primarily regulated by nongonadal factors.

Immunochemical evidence for neonatal printing of cytochrome P-450 has also been documented (Chao and Chung 1981).

1.3.3 HYPOTHALAMO-PITUITARY-LIVER AXIS

Several groups have independently reported that hypophysectomy of adult male and female rats resulted in the abolition of the sex differences observed in hepatic drug and steroid metabolism between males and females and enzyme activities returned to prepubertal levels (Denef 1974, Gustafsson and Stenberg 1974a, Kramer et al 1975a, Kamataki et al 1985). This effect was more prominent in adult female

rats, where hypophysectomy resulted in a marked decrease in the female-specific 5 α -reductase activity and an increase in male-specific oxidations. Thus hypophysectomy of female rats resulted in a "masculinisation" of steroid metabolism, indicating that the female pattern of steroid metabolism was dependent on the presence of an intact pituitary (Gustafsson and Stenberg 1974a). Further, the effects of the gonadal hormones were not observed in hypophysectomised rats (Gustafsson and Stenberg 1974d, Kramer et al 1975b, Colby et al 1973, Lax et al 1974), but unaffected by either adrenalectomy or thyroidectomy (Kramer et al 1979), thus indicating that the presence of the pituitary gland was essential for mediating gonadal hormone action. In addition, the absence of androgen receptors in the liver also supports an indirect action of androgens (Gustafsson et al 1976).

Implantation of an ectopic pituitary (secretes PRL and GH) under the kidney capsule of hypophysectomised male and female rats resulted in a feminisation of hepatic steroid metabolism (Eneroth et al 1977, Gustafsson and Skett 1978), suggesting that the pituitary gland may be releasing a "feminising factor" that is normally secreted in females only, and results in the feminisation effect observed. The administration to cultured hepatoma cells of a pituitary extract containing the feminising factor also resulted in the feminisation of hepatic steroid metabolism (Skett et al 1978, Gustafsson et al 1975).

Neither the liver nor the pituitary are sexually differentiated and so it was postulated that the control centre responsible for regulating the sex differences in

hepatic drug and steroid metabolism resided in higher centres of the CNS. Gustafsson and coworkers, in a series of articles, revealed that the hypothalamus was intricately involved in the regulation of this system via the hypothalamo-pituitary-liver axis (reviewed by Gustafsson et al 1980).

1.3.4 ROLE OF PITUITARY HORMONES

The addition of purified pituitary hormones exhibited a variety of effects. Firstly, FSH administration resulted in a partial masculinisation of steroid metabolism (Gustafsson and Stenberg 1975a), whereas treatment with LH and PRL (Gustafsson and Stenberg 1975b) had little effect. In contrast, Lax et al (1976) reported a decrease in reductive metabolism (masculinisation) in response to PRL. Colby et al (1974) using a combination of pituitary hormones showed that although FSH, LH, PRL, ACTH or GH administered to hypophysectomised rats had no effects on corticosterone metabolism, a combination of ACTH and GH significantly reduced reductase activity. In sharp contrast to this, however, Kramer and Colby (1976) have reported an increase in reductive metabolism upon treatment with GH alone.

GH administered to normal male rats decreased ethylmorphine and aniline metabolism, whilst no effects were observed in female rats (Kramer et al 1975a). In contrast, GH treatment of hypophysectomised rats of either sex increased ethylmorphine metabolism without altering either the rate of aniline metabolism or hepatic cytochrome P-450

content.

1.3.5 ROLE OF GROWTH HORMONE

Wilson (1968) reported a decrease in hepatic drug metabolism in response to growth hormone, probably due to a decrease in cytochrome P-450 content and NADPH cytochrome P-450 reductase activity (Wilson 1973). It was further demonstrated that the effect of growth hormone was not dependent on the gonadal status of the animal (Wilson 1971).

As was discussed in the previous section, a pituitary feminising factor is thought to be involved in maintaining the female pattern of steroid metabolism (Gustafsson et al 1975, Skett et al 1978). The identity of this feminising factor is now thought to be growth hormone (GH) (Rumbaugh and Colby 1980, Mode et al 1981, Skett and Young 1982), rather than a novel pituitary hormone. In fact, purification of the pituitary feminising factor showed the presence of growth hormone in the extract (Mode et al 1983).

The difficulty in the recognition of GH as the elusive pituitary feminising factor arose due to the similarity of MEAN serum GH concentrations in male and female animals. Edén (1979) showed that GH is secreted in a pulsatile manner in adult male rats, peaking every 3-4 hours with intervening low or undetectable levels. In contrast, GH is secreted in a more continuous manner in the female, and with a higher baseline. Prepubertal GH secretion is not sexually differentiated and the characteristic GH secretion profiles develop at the onset of puberty. This sex difference in the

pattern of GH secretion in adult rats is thought to dictate the sex-specific pattern of hepatic steroid metabolism in adult rats. Although sex differences in drug metabolism in mice exist, these are "reversed" as compared to the situation in rats. Nevertheless, GH also appeared to be the regulatory agent maintaining these sex differences in mice (MacLeod and Shapiro 1989).

Rumbaugh and Colby (1980) have subsequently reported that GH mediated the effects of oestradiol on hepatic drug and steroid metabolism, and further observed a feminisation effect in response to twice daily injection of GH in hypophysectomised animals. This effect was, however, only observed in the presence of ACTH and T_4 , suggesting that interactions with other hormones may also be important.

Mode et al (1981) demonstrated that continuous administration of human GH (hGH) by Alzet osmotic minipumps to hypophysectomised rats caused a complete feminisation of hepatic steroid metabolism. However, in contrast to the results of Rumbaugh and Colby (1980), these authors found that feminisation was still achieved in hypophysectomised, adrenalectomised, thyroidectomised and castrated male rats, thus excluding the dependency on the secretions of these glands. The discrepancy between the two reports can be partially accounted for since the method of GH administration was different (intermittent or continuous).

Several workers have reported similar results to the above, that is, continuous GH administration resulted in the feminisation of hepatic steroid metabolism whilst intermittent GH administration resulted in the expression of

male-specific and abolition of female-specific enzyme activities (Kato et al 1986, Mode et al 1982).

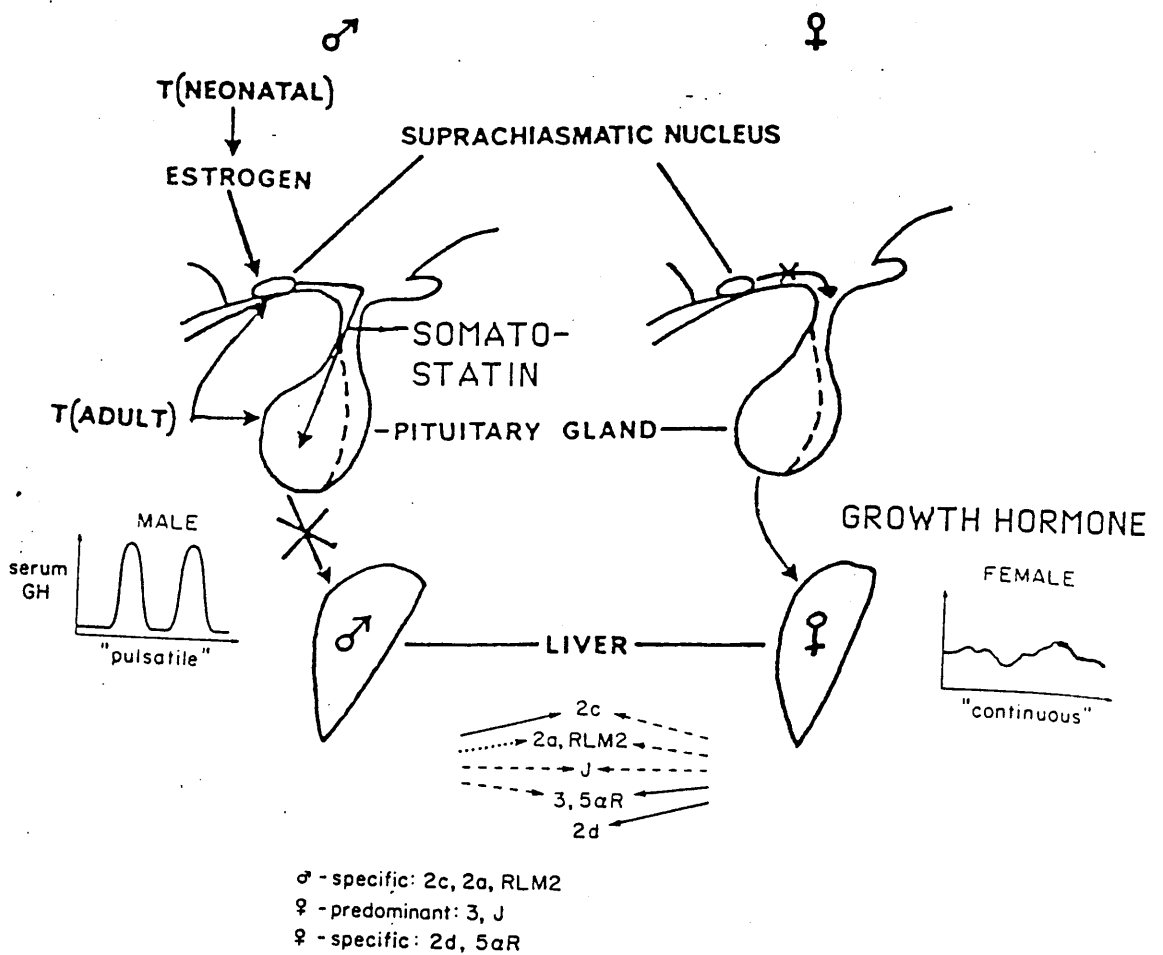
Jansson et al (1985) have proposed that neonatal androgens imprint the high amplitude pulses characteristic of GH secretion in adult male rats. Further, the role of the GH secretory pattern on the regulation of male- and female-specific activities has been implicated. Thus the female-specific 5 α -reductase activity was shown to be regulated by the GH secretory pattern (Miller and Colàs 1982), as was the expression of 15 β -hydroxylase (MacGeoch et al 1985) and 16 α -hydroxylase (Morgan et al 1985b) activities. GH was also thought to act as a repressive factor for testosterone 6 β -hydroxylation (Yamazoe et al 1986).

Since GH is thought to be the pituitary feminising factor, Norstedt et al (1983) have suggested that the factor released from the hypothalamus could be the growth hormone release inhibitory factor, somatostatin. The authors showed that both specific hypothalamic lesions and anti-somatostatin reduced somatostatin levels in the brain, together with decreases in microsomal steroid 6 β - and 16 α -hydroxylases. It was therefore suggested that somatostatin maintained the male pattern of steroid metabolism, and that the absence of this inhibitory influence in females led to the secretion of growth hormone and a feminisation of steroid metabolism.

Figure 2 summarises the proposed model for the imprinting and regulation of the rat hepatic drug- and steroid-metabolising system.

FIGURE 2 : NEONATAL IMPRINTING OF HEPATIC STEROID METABOLISING ENZYMES.

(adapted from Skett and Gustafsson 1979)



1.3.6 ROLE OF GLUCOCORTICOIDS

Remmer (1958) first demonstrated that adrenalectomy decreased hepatic microsomal oxidative activities in rats, an effect that could be fully reversed upon administration of glucocorticoids. Kato and Gillette (1965) then reported a sex difference in the effects of adrenalectomy on hepatic drug metabolism. Androgen-dependent substrates were metabolised more slowly in males, whereas no changes on hepatic metabolism were observed upon adrenalectomy in females.

Kato and Gillette (1965) subsequently showed that the metabolism of aminopyrine and hexobarbital by male rat liver microsomes was markedly decreased upon adrenalectomy whereas aniline and zoxazolamine metabolism was relatively unaffected. In contrast, adrenalectomy exerted no changes in drug metabolism in adult female rats, and so the authors suggested a possible permissive role of these hormones in mediating the actions of androgens in the male. They further suggested that this decrease in metabolism in male-specific activities (aminopyrine and hexobarbital) was as a consequence of a reduced binding capacity of cytochrome P-450 for hexobarbital and aminopyrine (Kato et al 1971), probably due to an impairment of the action of androgens to increase the binding capacity of cytochrome P-450 for these substrates.

Adrenalectomy resulted in a decrease in male-specific enzyme activities (Gustafsson and Stenberg 1974d) and in 17β -oxosteroid oxidoreductase activity, together with an

increase in 5 α -reductase activity (Lax et al 1979), thus reinforcing the results of Kato and coworkers described above.

Colby et al (1973, 1974) reported that administration of adrenal steroids had little effects on the 5 α -reduction of corticosterone and testosterone, since neither adrenalectomy nor corticosteroid treatment exerted any effects on enzyme activity. Treatment with the pituitary hormone adrenocorticotropin (ACTH), which stimulates the release of corticosteroids from the adrenal glands, did not affect the reduction of corticosterone (Colby et al 1974). However a combination of ACTH and GH was reported to markedly decrease the reduction of corticosterone, showing that hormonal interactions may be important in this system.

The actions of corticosteroids on hepatic drug metabolism were demonstrated in gonadectomised (Ichii and Yago 1969) and hypophysectomised rats (Bousquet et al 1965), indicating that their effects are not mediated by androgenic or pituitary factors. Similarly the gonadal hormones exhibited an effect in adrenalectomised rats (Kramer et al 1979, Mode et al 1981), showing that their effects are not dependent on the presence of intact adrenal glands.

From the effects of adrenalectomy on drug metabolism, the logical effect of glucocorticoid administration would be to increase hepatic metabolism. However, conflicting results have been reported with the effects of natural and synthetic glucocorticoids on this system. Tredger et al (1976) showed that whereas corticosterone reduced biphenyl hydroxylase and ethylmorphine N-demethylase activities, the synthetic

glucocorticoid, dexamethasone markedly increased these enzyme activities. Further, total cytochrome P-450 content was increased by dexamethasone treatment and unaffected by corticosterone. The author explained this discrepancy by showing that natural glucocorticoids such as corticosterone were rapidly metabolised in vivo whereas the synthetic glucocorticoids were more slowly metabolised, and therefore longer-acting. This does not however explain why corticosterone inhibited, while dexamethasone stimulated drug metabolism.

The induction profile of pregnenolone-16 α -carbonitrile (PCN) on drug-metabolising enzymes was first reported by Lu et al (1972) to be distinct from that of either phenobarbital or 3-methylcholanthrene (Horada and Omura 1981). Although PCN administration increased total cytochrome P-450 content by 2-fold, cytochrome P-450_{PCN} levels were induced more than 20-fold (Elshourbagy et al 1981, Heuman et al 1981). Dexamethasone, which gives a greater than 30-fold induction of cytochrome P-450_{PCN} appeared to be the most potent inducer of this isoenzyme (Schuetz et al 1984). Due to the relative inactivity of other steroids, together with the excessively high dose of dexamethasone required for P-450_{PCN} (P-450 IIIA1) induction as compared to other glucocorticoid functions such as stimulation of tyrosine aminotransferase, it was proposed that dexamethasone was acting by a mechanism distinct from that of the classical glucocorticoid receptor pathway (Schuetz and Guzelian 1984). In addition, the dexamethasone-induced increases in P-450_{PCN} were unaffected

in the presence of glucocorticoid antagonists that abolished tyrosine aminotransferase activation (Schuetz et al 1984).

Glucocorticoids are known to influence a number of components of the hepatic drug- and steroid-metabolising system. A reduction in NADPH-cytochrome P-450 reductase activity (Castro et al 1970) and a decrease in cytochrome P-450 levels (Yago and Ichii 1969) was reported following adrenalectomy. Hamrick et al (1973) showed that administration of cortisone acetate resulted in an increase in NADPH cytochrome c reductase, but cytochrome P-450 levels were reduced. Further, Tredger et al (1976) reported that whilst the synthetic glucocorticoid dexamethasone stimulated both of the above parameters, corticosterone exhibited no such effects.

Adrenalectomy has also been reported to increase haem oxygenase activity, and thus the degradation of cytochrome P-450 (Sardana et al 1980). This would explain the reduction in cytochrome P-450 content in response to adrenalectomy.

1.3.7 ROLE OF THYROID HORMONES

The thyroid hormones are known to influence the metabolism of drugs and steroids by the hepatic cytochrome P-450 system. A great deal of work has been carried out by Kato and coworkers in this respect.

Kato and Gillette (1965) reported that the effects of thyroxine on drug metabolism exhibited both sex- and substrate-dependent effects in rats. Whereas metabolism of the androgen-dependent substrates aminopyrine and

hexobarbital was impaired by thyroxine in adult male rats, metabolism of the sex-independent substrate aniline was slightly increased. Drug metabolism was enhanced upon thyroxine administration to female rats.

The effects of thyroxine on steroid metabolism were also sex-dependent and reported to be similar to those on drug metabolism. Thyroxine administration to adult male rats resulted in a decrease in the hydroxylation of progesterone (Kato et al 1970b) and testosterone (Kato et al 1970a), and increased progesterone and hydrocortisone reduction (Kato et al 1970c). Both cytochrome P-450 content and substrate interaction with cytochrome P-450 were reduced upon treatment with thyroxine. In contrast, thyroxine exhibited no effects in females, except for a slight decrease in the reductive metabolism of testosterone and hydrocortisone.

Thyroidectomy of male rats (Kato et al 1970b) resulted in a decrease in the hydroxylation of testosterone and progesterone, but no effects were observed on either reductase activity or upon thyroidectomy of female rats. Thyroidectomy also had no apparent effects upon either cytochrome P-450 content or on substrate interaction with cytochrome P-450 in females.

Both thyroxine and thyroidectomy were reported to influence several components of the cytochrome P-450 system. Kato and Takahashi (1968) showed that thyroxine increased NADPH oxidase and NADPH cytochrome c reductase activities in male and female rats, although the increase was reported to be greater in females. Cytochrome P-450 content was only increased in male rats.

The metabolism of aminopyrine, hexobarbital and aniline, and the activities of NADPH oxidase, NADPH cytochrome c reductase and NADH oxidase were decreased upon thyroidectomy of male and female rats, although NADH cytochrome c reductase, cytochrome b₅ and P-450 content remained unchanged. The administration of triiodothyronine to thyroidectomised females was reported to reverse all the effects of thyroidectomy, but cytochrome P-450 content and drug metabolism remained low in males.

Studies of cytochrome P-450 binding spectra (Kato et al 1970a) led to the conclusion that the reason for the reduction in the metabolism of aminopyrine and hexobarbital in male rats in response to thyroxine administration was due to an impaired binding capacity of cytochrome P-450 for these substrates, together with the direct effects of thyroxine observed on cytochrome P-450 content and binding capacity, and other components of this system (Kato and Takahashi 1968). A similar explanation was offered for the effects of thyroidectomy.

The relative specificity of thyroxine and thyroidectomy for male-specific functions suggested an involvement of androgens in this system. The authors therefore suggested that the actions of thyroxine may involve the gonadal hormones, since the effects of thyroxine were abolished in castrated rats, but restored in androgen-treated castrates (Kato and Gillette 1965). Further, the effects of thyroxine in androgen-treated females were reported to be identical to those in males (Kato et al 1970c), indicating a dependency of androgens in the actions of thyroxine. Hence the effects

of thyroxine on steroid and drug metabolism may be due to an impairment of androgen action. In addition, Colby et al (1973) showed that thyroxine failed to affect steroid metabolism in orchietomised rats. On the basis of these findings, the authors concluded that thyroxine antagonised the stimulatory effects of androgens on hepatic microsomal metabolism, but in the absence of androgens, thyroxine increased hepatic metabolism.

The studies carried out by Kato's group, have, however been disputed by several authors due to their use of thyrotoxic doses of the thyroid hormones. Rumbaugh et al (1978) showed that administration of physiological doses of thyroxine stimulated drug metabolism in hypophysectomised male and female rats, whereas larger doses of thyroxine inhibited metabolism of some substrates in a sex-dependent manner. In addition, thyroxine produced marked dose-dependent decreases in hepatic cytochrome P-450 content and increases in NADPH cytochrome c reductase activity in hypophysectomised males and females (Colby and Rumbaugh 1985). Thus the effects of physiological doses of thyroxine do not appear to correlate with changes in cytochrome P-450 levels (although specific isoenzymes may have increased), but rather follow the changes in NADPH cytochrome c reductase activity.

Under normal circumstances, the stimulatory effects of physiological doses of thyroxine are in agreement with the finding that thyroidectomy results in a decrease in drug metabolism. In addition, numerous clinical reports have shown that hypothyroidism increased while hyperthyroidism

decreased the plasma half-life of drugs (Vesell et al 1975, Saenger et al 1976).

That the effects of thyroxine were exhibited in hypophysectomised rats shows that thyroxine acts directly on the liver, and not via pituitary hormones. In contrast to the conclusions of Kato, Colby and Rumbaugh (1985) proposed that the effects of thyroxine were not dependent on the gonadal status of the animal, and that thyroxine stimulated hepatic metabolism irrespective of the presence or absence of oestradiol and androgen.

In dispute with the above results, Skett and Weir (1983) demonstrated sex- and substrate-dependent effects of physiological doses of thyroxine, and further showed that thyrotoxic doses resulted in a marked decrease in cytochrome P-450 content in males together with the abolition of the sex-dependent effects observed on drug metabolism. In addition, thyroidectomy was also reported to exhibit sex differences in drug metabolism, and replacement thyroxine treatment of thyroidectomised rats reversed some effects of thyroidectomy whilst enhancing others. The existence of sex differences in steroid metabolism in response to thyroidectomy has previously been documented (Lax et al 1979).

Skett and Weir (1983), in an attempt to explain the discrepancies in the effects of thyroxine, suggested that an optimal concentration of thyroxine existed for exhibiting maximal effects on hepatic metabolism, and that deviations from this optimum, either in the direction of hypo- or hyperthyroidism, resulted in loss of enzyme activity.

More recently, it has been suggested that the thyroid hormones may function as regulatory hormones in the expression of constitutive levels of cytochrome P-450_a (gene IIA1), catalysing the 7 α -hydroxylation of testosterone (Arlotto and Parkinson 1989) since thyroidectomy resulted in an increase in cytochrome P-450_a (gene IIA1) expression that was restored upon treatment with thyroid hormones.

Previous studies have already documented a role of the thyroid hormones in the regulation of liver microsomal cytochrome b₅, NADPH cytochrome P-450 reductase and haem oxygenase (Phillips and Langdon 1962, Hoch et al 1980, Smith et al 1982, Kriz et al 1982).

1.3.8 ROLE OF INSULIN

The role of insulin in the regulation of cytochrome P-450-dependent drug and steroid metabolism has been indirectly implicated by the marked effects of both spontaneous and chemically-induced diabetes.

Dixon et al (1961) first reported that alloxan-induced diabetes in male rats decreased the metabolism of hexobarbital, codeine and chlorpromazine in vitro and prolonged hexobarbital sleeping time in these animals. Subsequent studies have confirmed this initial report. Kato and Gillette (1965) showed that the metabolism of aminopyrine and hexobarbital by male rat liver microsomes was impaired upon treatment with alloxan, while aniline metabolism was enhanced. In contrast, the metabolism of these substrates was enhanced in alloxan-treated females.

The authors reported that the effects of alloxan were only evident in the presence of androgens, which may explain why alloxan only affected sex-dependent substrates such as aminopyrine and hexobarbital. It was subsequently suggested in a further report (Kato et al 1970e) that the stimulant effect of androgens on oxidative metabolism in male rat liver microsomes was blocked by alloxan or starvation, resulting in a decrease in drug oxidations. This was further supported by the observation that metabolism of the androgen-independent substrate aniline was not decreased by alloxan in male rats, or in female rats. In addition, Kato et al (1970f) demonstrated that the binding capacity of cytochrome P-450 for androgen-dependent substrates in male rats was decreased by alloxan, and so the authors postulated that this decrease was as a direct consequence of an impairment of the ability of androgen to stimulate the binding capacity of cytochrome P-450 with these substrates.

Several groups have documented that the effects of alloxan on drug metabolism may be due to its toxicity rather than diabetogenic effects (Hoftiezer and Carpenter 1973). Streptozotocin (STZ), a selective β -cell toxin was subsequently used to induce diabetes in experimental animals, and was reported to cause a decrease in hexobarbital and an increase in aniline metabolism (Ackerman and Liebman 1977). Since these effects were reversible upon insulin therapy, it was concluded that STZ was exerting diabetogenic effects on drug metabolism.

The sex-dependent effects of STZ-induced diabetes have more recently been demonstrated by Skett and Joels (1985).

Whereas male-specific enzyme activities responsible for the metabolism of diazepam, lignocaine and imipramine were decreased, non-sex-specific enzyme activities were unaffected in male rats. Further, a time-dependency was also observed in the effects of STZ on drug metabolism in that acutely (3-day) STZ-treated rats exhibited all of the above effects whereas no changes were observed in chronically (21-day) rats. In contrast, STZ elicited no changes in drug metabolism in females.

Skett (1986) subsequently showed that the effects of STZ treatment on hepatic steroid metabolism were sex-dependent and similar to those observed with drug metabolism. The effects of diabetes were only observed in the male, and exhibited an abolition of the sex differences observed in hepatic steroid metabolism. Female-specific activities were increased and male-specific activities decreased by diabetes, which is characteristic of the feminisation effect observed with continuous growth hormone infusion (Mode et al 1981). Since insulin-deficient conditions led to the feminisation of steroid metabolism, it was proposed that insulin may be exerting a "masculinising" influence on hepatic metabolism in vivo (Skett 1986).

Insulin administration to isolated rat hepatocytes resulted in a general stimulation of hepatic steroid-metabolising enzymes (Hussin and Skett 1987) without altering total cytochrome P-450 content. The possibility of the stimulation of individual isoenzymes was, however, not examined. It was further shown that, whereas hepatocytes from 3- and 21-days STZ-diabetic rats were resistant to the

stimulatory effects of insulin (Hussin and Skett 1988), insulin-treated diabetics indicated only a partial restoration of the response with insulin, implying that other factors may also be important for the restoration of the response to insulin.

Skett et al (1984) showed that the effects of STZ-diabetes and castration in the male rat were similar, and found that STZ had little effects in castrated rats. Further, the effects of STZ in castrated rats could be restored upon treatment with androgen, suggesting a certain degree of interaction between the two effects. The results on drug metabolism in this study did not however, correlate with changes in serum testosterone. The involvement of androgens in the diabetic state has also been suggested by Kato and Gillette (1965). It was therefore proposed that there was an interaction between androgens and insulin in this system that was mediated via a common mediator, growth hormone (Skett et al 1984). Tannenbaum (1981) has shown that STZ-diabetes resulted in marked depression of the pulsatile GH secretory pattern in male rats, and further suggested a role of somatostatin in this effect. Certainly, the somatotrophs of diabetic rats are relatively resistant to somatostatin, as well as elevated growth hormone-releasing hormone levels (Welsh and Szabo 1988). It has further been suggested that the effects of diabetes on the growth hormone secretory pattern may be as a consequence of the low circulating levels of thyroid hormone in these animals (Ortiz-Caro et al 1984), since growth hormone levels were decreased in thyroidectomised rats (Coiro et al 1979).

The effects of diabetes at the biochemical level have been documented by numerous authors, and a diabetes-dependent form of cytochrome P-450 has been isolated. Past and Cook (1982) isolated a 52K cytochrome P-450 by SDS-PAGE from the microsomal fraction of alloxan-treated diabetic rats. They showed that this band was not present in control, 3-methylcholanthrene- or phenobarbital-treated rats, or from insulin-treated diabetic rats. It was therefore suggested that the increase in this form of cytochrome P-450 in diabetes was responsible for the increased metabolism of aniline (Kato and Gillette 1965).

More recently, Bellward et al (1987) have demonstrated increased levels of cytochrome P-450_j (gene IIE1) in spontaneously diabetic BB rats, as well as increases in cytochrome P-450_j-catalysed activities, aniline hydroxylation and N-nitrosodimethylamine N-demethylation. These effects were all reversible upon replacement insulin therapy. In contrast, cytochrome P-450_f (gene IIC7) levels remained unchanged between diabetic and non-diabetic conditions.

It has recently been reported that levels of cytochrome P-450 forms RLM3 (gene IIC13) and RLM5 (gene IIC11) were markedly decreased, and RLM5b and RLM6 (gene IIE1) increased within three weeks of the onset of STZ diabetes, and restored by insulin therapy (Favreau and Schenkman 1988a), whereas RLM5a (gene IIC6) levels remained unchanged.

The results observed with STZ-induced diabetics were later confirmed in a subsequent study carried out in spontaneously diabetic rats (Favreau and Schenkman 1988b).

The induction of cytochrome P-450_{ac} (gene IIE1) was also reported in STZ-diabetic, alloxan-diabetic and BB/Wor rats (Dong et al 1988), which correlated well with the observed increase in P-450_{ac} mRNA, possibly as a result of mRNA stabilisation and enhanced protein stabilisation due to the high level of acetone produced (Song et al 1987). Further, it has recently been shown that both actinomycin D (inhibits RNA synthesis) (Peng et al 1983) and cycloheximide (inhibits protein synthesis) (Tu et al 1983) reduced the effects of fasting on drug metabolism, and so diabetes may be exerting its effects in a similar manner.

Experimentally-induced diabetes has also been reported to reduce the expression of the inhibitory G-protein (G_i) (Gawler et al 1987). Since insulin is thought to interact with G-proteins during its action (Houslay et al 1989), the impairment of many of the effects of insulin in diabetes may be as a direct consequence of this effect.

1.3.9 SEX-DEPENDENCY AND DEVELOPMENTAL REGULATION OF CYTOCHROME P-450

Although the lower level of total microsomal cytochrome P-450 protein in the female as compared to the male rat is adequate to explain the overall slower metabolism of xenobiotics in female rats, it does not explain the large sex differences obtained in hepatic metabolism. The metabolism of endogenous steroids, such as androst-4-ene-3,17-dione, is sex-specific in nature in that certain enzymes display higher activity in male rats while

others are more active in females (Table 1).

TABLE 1 : SEX-SPECIFIC METABOLISM OF TESTOSTERONE AND ANDROST-4-ENE-3,17-DIONE (Gustafsson and Stenberg 1974)

<u>ENZYME</u>	<u>SEX-SPECIFICITY</u>
5 α -reductase	male < female
3 α -hydroxysteroid dehydrogenase	male = female
3 β -hydroxysteroid dehydrogenase	male > female
17 α -hydroxysteroid dehydrogenase	male = female
17 β -hydroxysteroid dehydrogenase	male > female
6 β -hydroxylase	male > female
7 α -hydroxylase	male \leq female
16 α -hydroxylase	male > female
steroid sulphate 15 β -hydroxylase	male < female

Sex-related differences in the metabolism of drugs and steroids have now been attributed to the presence of male- and female-specific isoenzymes of cytochrome P-450 (Lu and West 1980, Morgan et al 1985b).

Recently, a number of groups have isolated and purified various forms of cytochrome P-450 (Waxman 1984, Kamataki et al 1983, Morgan et al 1985b, Funae and Imaoka 1987) which would explain these sex-differences observed in hepatic metabolism. Kamataki et al (1981) isolated two forms of cytochrome P-450 from male and female rats, which they termed cytochrome P-450-male and cytochrome P-450-female respectively. Cytochrome P-450-male metabolised aminopyrine and benzphetamine more efficiently than the female form, whereas ethylmorphine was metabolised to an equal extent by both forms (Kamataki et al 1981). Further, using specific antibodies against these cytochrome P-450 forms, they showed

that cytochrome P-450-male was only present in male rats, and that its levels were markedly reduced upon castration and restored to normal levels upon treatment with testosterone. Similarly cytochrome P-450-female was only detected in female rats and abolished upon ovariectomy.

Kamataki et al (1985) have recently studied the developmental profile of these two forms and have shown that both cytochromes P-450-male and P-450-female are virtually undetectable prepubertally in male and female rats. However, the onset of puberty at approximately 25 days of age was shown to parallel the appearance of cytochromes P-450-male and P-450-female in male and female rats respectively, and correlated well with the changes observed in hepatic drug and steroid metabolism.

Male-specific enzymes have also been isolated by numerous other groups. Morgan et al (1985a) isolated cytochrome P-450 RLM5 (gene IIC11) from male rats, and correlated this with catalysing the hydroxylation of steroids in the 16 α position. Further, it was shown in the same study and by Pasleau et al (1981) that the expression of cytochrome P-450_{16 α} (gene IIC11) was only observed in the male after the onset of puberty, and not in prepubertal male or female rats.

Immunochemical studies have shown the existence of cytochromes P-450_{2c/UT-A} (gene IIC11) in adult male rat liver and P-450_{2d/UT-1} (gene IIC12) in adult females (Waxman 1984). Characterisation of these forms of cytochrome P-450 (Waxman et al 1985) showed that the male-specific cytochrome P-450_{2c/UT-A} (gene IIC11) was the major microsomal steroid

16 α -hydroxylase of uninduced male rat liver, and activity was increased about 30-fold at the onset of puberty. Similarly, cytochrome P-450_{2d/UT-1} (gene IIC12) was developmentally induced in female rats at puberty, corresponding with an increase in 15 β -hydroxylase activity (Waxman et al 1985, MacGeoch et al 1984). Postpubertal castration of male animals did not affect cytochrome P-450_{15 β} (gene IIC12) levels, whereas neonatal castration significantly increased cytochrome P-450_{15 β} (gene IIC12) content. Administration of oestradiol valerate also feminised male cytochrome P-450_{15 β} (gene IIC12) levels. From this it was concluded that cytochrome P-450_{15 β} (gene IIC12) is suppressed by neonatal androgen and is therefore not expressed in male rats (MacGeoch et al 1985).

Cytochrome P-450_{2a/PCN-E} (gene IIIA2), catalysing $\geq 85\%$ of microsomal steroid 6 β -hydroxylation (Waxman et al 1985) was also male-specific. This was, however, due to the suppression of 6 β -hydroxylase activity in mature females rather than an induction in mature males rats at puberty.

Neonatal gonadectomy and hormone replacement studies have established that neonatal androgen is responsible for the imprinting of cytochrome P-450_{2c} (gene IIC11), for the maintenance of cytochrome P-450_{2a} (gene IIIA2), and for the suppression of cytochrome P-450_{2d} (gene IIC12) in male rats (Waxman et al 1985).

The female-specific enzyme microsomal 5 α -reductase was also expressed significantly in female rats at puberty (Waxman et al 1985). The regulation of the two female-specific enzymes 5 α -reductase, and 15 β -hydroxylase,

has been studied in greater detail by Dannan et al (1986), who showed that, although enzyme activities were unaffected by oestrogen treatment, postpubertal androgen administration resulted in a marked suppression of the two activities.

Cytochrome P-450_{3/UT-F} (gene IIA1) and its corresponding steroid 7 α -hydroxylase activity was present at much higher levels in adult females than males (Waxman et al 1985). This was thought to be due to the rapid decrease in expression in male rats rather than an induction in female rats at puberty. Guengerich et al (1982) have reported a similar age-dependent cytochrome P-450 (P-450_{PB-1/PB-C}) (gene IIC6) catalysing warfarin 7 α -hydroxylase activity, which was present in both uninduced male and female rats.

1.4 STEROID METABOLISM

1.4.1 CYTOCHROME P-450 NOMENCLATURE

Since the original idea proposed by Lu and West in 1980, that the cytochromes P-450 exist in the form of isoenzymes with distinct yet overlapping substrate specificities, numerous authors have subsequently isolated, purified and characterised a whole spectrum of cytochrome P-450 isoenzymes.

Isoenzymes isolated by different authors have been designated using their own nomenclature systems, resulting in a collection of names for each isoenzyme.

Nebert and Gonzalez (1987) have subsequently introduced a universal nomenclature system for different isoenzymes

based on their primary amino acid sequences. The cytochromes P-450 are grouped into a gene superfamily, which is subdivided into gene families and into gene subfamilies.

Gene families are designated by Roman numerals, and currently characterised hepatic drug-metabolising enzymes are categorised into gene families I to IV. Extrahepatic cytochromes P-450 catalysing steroid biosynthesis have been classified into gene families XVII (steroid 17 α -hydroxylase), XIX (cytochrome P-450_{aromatase}) and XXI (steroid 21-hydroxylase). The mitochondrial, yeast and bacterial cytochrome P-450 gene families are referred to as XI, LI/LII and CI/II respectively. Gene subfamilies are denoted by capital letters, followed by Arabic numbers to identify genes within a subfamily. Gaps have been left throughout to allow for new cytochromes that may be isolated. The nomenclature system of Nebert and Gonzalez (1987) is summarised in Table 2.

TABLE 2 : HEPATIC CYTOCHROME P-450 NOMENCLATURE (adapted from Nebert and Gonzalez 1987, Gonzalez 1989)

<u>Family/subfamily</u>	<u>Characterisation</u>
P-450I (polycyclic aromatic compound inducible)	
P-450IA1	Rat c, rabbit 6, mouse P ₁ , human P ₁
P-450IA2	Rat d, rabbit 4, mouse P ₃ , human P ₃
P-450II (major)	
P-450IIA subfamily	
P-450IIA1	Rat a
P-450IIA2	RLM2
P-450IIA3	Human P-450(1), mouse 15a
P-450IIB subfamily (phenobarbital-inducible)	
P-450IIB1	Rat b, rabbit 2, human MP
P-450IIB2	Rat e
P-450IIB3	Rat
P-450IIB4	Rabbit LM2
P-450IIB5	Rabbit HP1
P-450IIB7	Human
P-450IIB8	Human
P-450IIB9	Mouse pf26
P-450IIB10	Mouse pf3/46
P-450IIC subfamily	
P-450IIC1	Rabbit PBc1
P-450IIC2	Rabbit PBc2, K, pHP2
P-450IIC3	Rabbit PBc3, form 3b
P-450IIC4	Rabbit PBc4, form p1-88
P-450IIC5	Rabbit 1
P-450IIC6	Rat PB1, PB-C, k
P-450IIC7	Rat f
P-450IIC8	Human form 1
P-450IIC9	Human MP-4
P-450IIC10	Chicken PB15, Human MP-8
P-450IIC11	Rat M-1, h, 2c, 16a
P-450IIC12	Rat i, 2d, 15β
P-450IIC13	Rat g
P-450IIC14	Rabbit pHP
P-450IIC15	Rabbit b32-3
P-450IID subfamily	
P-450IID1	Rat db1, human db1
P-450IID2	Rat db2
P-450IID3	Rat
P-450IID4	Rat
P-450IID5	Rat
P-450IID6	Human db1
P-450IID7	Human
P-450IID8	Human
P-450IID9	Mouse 16a
P-450IIE subfamily (ethanol-inducible)	
P-450IIE1	Rat j, rabbit 3a, human j
P-450IIE2	Rabbit gene 2
P-450III (steroid-inducible)	
P-450IIIA1	Rat pcn1
P-450IIIA2	Rat pcn2
P-450IIIA3	Human p
P-450IIIA4	Human nf
P-450IIIA5	Human PCN

P-450IIIA6	Rabbit 3c
<u>P-450IV (peroxisome proliferator-inducible)</u>	
P-450IVA1	Rat LAw
<u>P-450XI (mitochondrial proteins)</u>	
P-450XIA subfamily	
P-450XIA1	Bovine and human scc
P-450XIB subfamily	
P-450XIB1	Bovine and human 11 β
<u>P-450XVII (steroid 17α-hydroxylase)</u>	
P-450XVIIA1	Bovine and human 17 α
<u>P-450XIX</u>	
P-450XIXA1	Human aromatase
<u>P-450XXI (steroid 21-hydroxylase)</u>	
P-450XXIA1	Bovine, mouse and human C21A
P-450XX1A2	Bovine, mouse and human C21B
<u>P-450LI (plant P-450)</u>	
P-450LIA1	Yeast Ian
<u>P-450CI (prokaryote P-450)</u>	
P-450CIA1	Pseudomonas putida cam

1.4.2 STRUCTURAL SIMILARITY OF CYTOCHROME P-450

ISOENZYMES

The cytochromes P-450 catalyse the metabolism of a diverse range of drugs and steroids owing to the presence of isoenzymes showing greater specificity towards certain substrates than others. Steroid hormones have proved to be very useful in this respect for distinguishing between these multiple isoenzymes since they often yield unique metabolic profiles (Figure 3). The most widely studied steroids have been androstenedione and testosterone (Woods et al 1983, Waxman et al 1983, Sheets and Estabrook 1985, Sonderfan et al 1987), and so the characterisation of cytochrome P-450 isoenzymes in this section will, for the greater part, be restricted to the metabolism of these two substrates. The recognition of the presence of multiple isoenzymes with unique yet overlapping substrate specificities was attributed to the presence of a family of structurally related cytochrome P-450 proteins (Lu and West 1980).

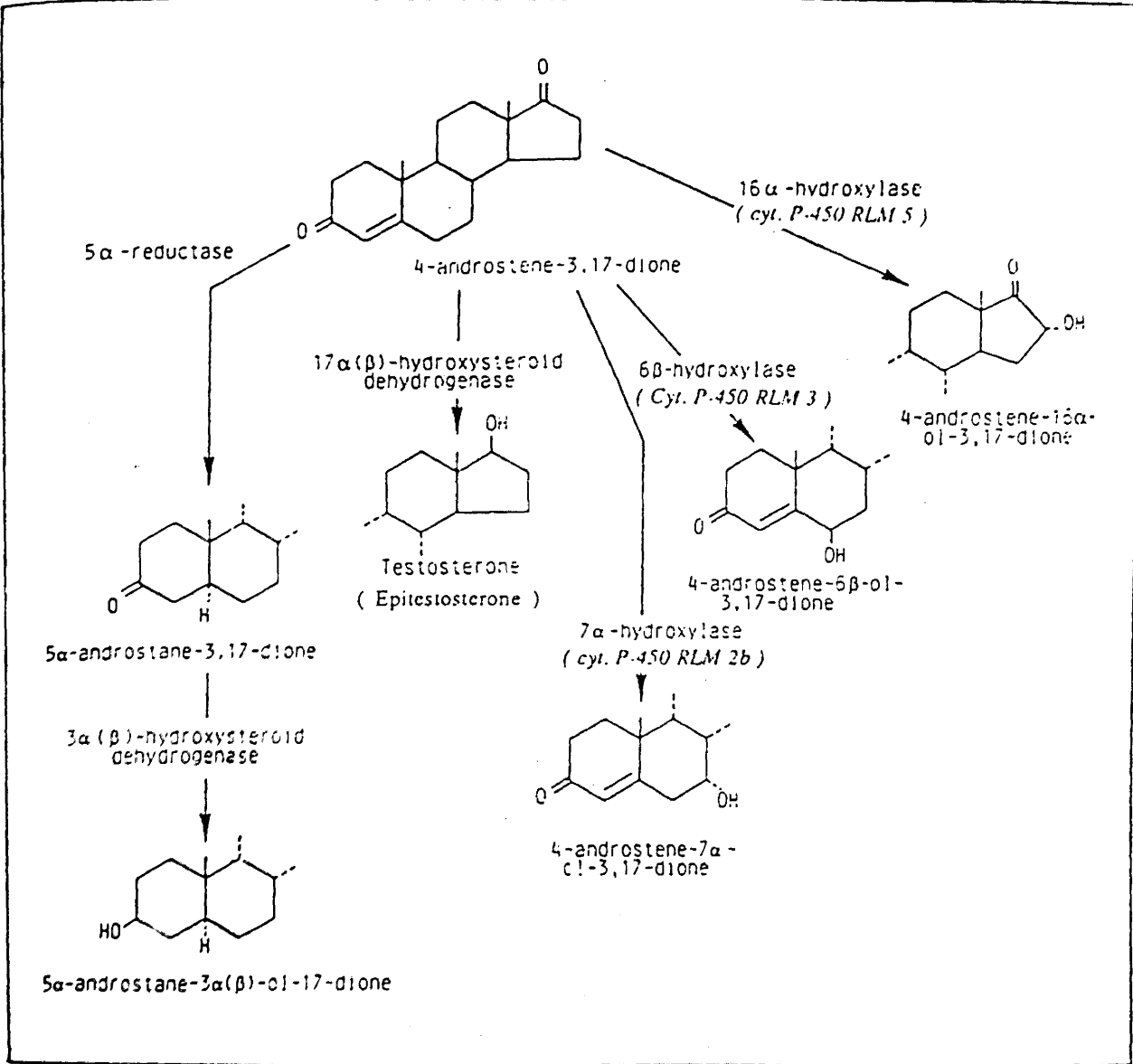
A variety of techniques have been employed in the identification of different isoenzymes :

i) Immunochemical cross-reactivity - useful in the determination of similarities between different isoenzymes, and also different species eg rat-human.

ii) Protein sequencing - Black and Coon (1986) have determined the amino acid sequences of a number of cytochromes P-450, which has subsequently led to the gene designations of Nebert and Gonzalez (1987).

iii) DNA sequencing - to determine similarities amongst

FIGURE 3 : METABOLITE PROFILE OF ANDROSTENEDIONE.



P-450 genes. In general, similarity is greater among P-450 genes in protein coding regions than in non-coding regions.

iv) Substrate specificity - eg Cytochromes P-450_{16a} (Pasleau et al 1981), P-450_{2c} (Waxman 1984) and P-450-male (Kamataki et al 1983) have been shown to catalyse the same hydroxylation reaction. Also important in the determination of major and minor reactions catalysed by a particular isoenzyme.

Although the nomenclature system used by the various authors will be sustained in the following discussion of their results, the gene designation given to that particular isoenzyme will also be given. Isoenzymes relevant to this study are discussed in detail, but all, together with their specific activities, are summarised in Table 3.

TABLE 3 : CYTOCHROME P-450 ISOENZYMES (modified from Waxman 1988)

Waxman	Levin	Guengerich	Schenkman	Wolf	other	GENE
PB-1	k	PB-C	RLM5a	PB1b	---	IIC6
PB-inducible warfarin 7 α -hydroxylase; hormone independent						
2a	---	----	-----	----	---	IIIA2
Male-specific testosterone 6 β -hydroxylase						
PB-2a	p	PCN-E	-----	----	---	IIIA1
Synthetic steroid-inducible testosterone 6 β -hydroxylase						
2c	h	UT-A	RLM5	PB2a	male	IIC11
Male-specific testosterone 2 α /16 α -hydroxylase						
2d	i	UT-1	fRLM4	---	female	IIC12
Female-specific steroid sulphate 15 β -hydroxylase						
3	a	UT-F	-----	UT1	---	IIA1
Steroid hormone 7 α -hydroxylase						
PB-4	b	PB-B	PBRLM5	PB3a	---	IIB1
Major phenobarbital-inducible testosterone 16 β -hydroxylase						
PB-5	e	PB-D	PBRLM6	PB3b	---	IIB2
97% homologous to PB-4 (IIB1)						
6	---	----	-----	----	---	----
Constitutive form, cross-reacts with PB-4 (IIB1)						
BNF-B	c	BNF-B	-----	MC1b	---	IA1
Major 3-methylcholanthrene-inducible form						
ISF-G	d	ISF-G	-----	MC1a	---	IA2
Major isosafrole-inducible form						
---	f	----	-----	----	---	IIC7
Constitutive form, cross-reactive with PB-4 (IIB1)						
---	g	----	RLM3	----	---	IIC13
Male-specific, strain dependent						
---	j	----	RLM6	----	---	IIE1
Ethanol-inducible nitrosamine demethylase						

1.4.3 RAT CYTOCHROME P-450 ISOENZYMES

a) 7 α -HYDROXYLASE (P-450 IIA1)

Ryan et al (1979) isolated cytochrome P-450_a (MW 48000) which has been reported to catalyse the 7 α -hydroxylation of testosterone, progesterone and androstenedione (Wood et al 1983, Waxman 1984, Waxman et al 1985, Nagata et al 1986). Arlotto and Parkinson (1989) have recently described the purification of two structurally related cytochrome P-450 isoenzymes, P-450_a (gene IIA1) and P-450_m, which catalysed the 7 α -hydroxylation of testosterone in a reconstituted system. Cytochrome P-450_a (gene IIA1) (Arlotto et al 1989) appeared to be solely responsible (>96%) for the 7 α -hydroxylation in rat liver microsomes, and cytochrome P-450_m only a minor pathway in testosterone oxidation. A similar form of cytochrome P-450 has been purified from dog liver microsomes (Duignan et al 1988).

Cytochrome P-450_a (gene IIA1) was present in one week old male rats (Arlotto and Parkinson 1989), and 7 α -hydroxylase activity appreciably declined with age. In contrast, 7 α -hydroxylase activity did not decline in mature female rats, resulting in higher levels of this activity in adult female than male rats (Waxman et al 1985).

Neonatal castration of males and neonatal exposure of females to androgens had little effects on cytochrome P-450_a (gene IIA1) levels, indicating that 7 α -hydroxylase activity is not regulated by neonatal androgen exposure, and is regulated by nongonadal factors (Waxman et al 1985).

Cytochrome P-450_a (gene IIA1) was reported to be

induced by 3-methylcholanthrene or phenobarbital (Guengerich et al 1982, Dannan et al 1983, Ryan et al 1984).

b) 16 α -HYDROXYLASE (P-450 IIC11)

Ryan et al (1984) isolated cytochrome P-450 forms f, g and h catalysing the hydroxylation of testosterone. Cytochromes P-450_f (gene IIC7) and P-450_h (gene IIC11) catalysed the 16 α -hydroxylation of testosterone. Cheng and Schenkman (1983) reported that the 16 α -hydroxylation of testosterone was a major activity of cytochrome P-450 RLM₅ (gene IIC11) in rat liver microsomes. Cytochrome P-450_{UT-A} (gene IIC11) also accounts for the minor pathway of 2 α -hydroxylation of testosterone in rat liver microsomes (Waxman 1984, Cheng and Schenkman 1983). This cytochrome P-450 form has also been purified by numerous other groups (Dannan et al 1986, Kamataki et al 1983, Mode et al 1981, Morgan et al 1985a, Ryan et al 1984, Waxman 1984), and exhibited steroid 16 α -hydroxylase activity.

Regulation of this isoenzyme has been extensively studied (Pasleau et al 1981), and it is now believed that cytochrome P-450_{2c} (gene IIC11) corresponds to the male-specific 16 α -hydroxylase activity of rat liver induced at puberty (Waxman 1984), and that its expression is imprinted by neonatal androgens (Dannan et al 1986). In addition, expression of cytochrome P-450-male, which was reported to be approximately 20-fold higher in adult male rats (Morgan et al 1985a), was decreased upon hypophysectomy (Kamataki et al 1985), and further decreased upon continuous infusion of GH into hypophysectomised male rats (Kato et al

1986). In contrast, intermittent GH administration into hypophysectomised males resulted in an increased expression of cytochrome P-450-male (Kato et al 1986). This implies that the level of expression of cytochrome P-450_{1 β a} (gene IIC11) is regulated by the secretory pattern of GH (Morgan et al 1985a, Ström et al 1988). Both neonatal and postpubertal castration of male rats decreased cytochrome P-450_{1 β a} (gene IIC11) levels, and these were restored by neonatal or postpubertal testosterone treatment (Kamataki et al 1985, Waxman et al 1985, Dannan et al 1986).

Administration of β -naphthoflavone, phenobarbital, PCN and isosafrole led to the suppression of cytochrome P-450_{2c} (gene IIC11) and its associated 16 α -hydroxylase activity (Waxman et al 1985, Guengerich et al 1982) and was virtually abolished by polybrominated biphenyl compounds (Dannan et al 1983). Decreases in the expression of male-specific forms of cytochrome P-450 by inducers, and therefore abolition of sex differences in drug and steroid metabolism, have been documented extensively (Kamataki et al 1986, Emi and Omura 1988, Shimada et al 1989).

c) 6 β -HYDROXYLASE (P-450 IIIA2)

The major isoenzyme responsible for the 6 β -hydroxylation of testosterone in a reconstituted system was shown to be cytochrome P-450 RLM3 (gene IIC13) (Cheng and Schenkman 1983) and cytochrome P-450 PB-1 (gene IIC6) (Imaoka et al 1988). Cytochrome P-450_{2a/PCN-E} (gene IIIA2) was reported to mediate >85% of the 6 β -hydroxylation of testosterone (Waxman et al 1985).

Significant levels of cytochrome P-450_{6β} (gene IIIA2) are present in males irrespective of age of the animal. In contrast, cytochrome P-450_{6β} (gene IIIA2) levels decreased markedly in the female at the onset of puberty (Waxman et al 1985).

Neonatal androgen secretion was reported to play an essential role in imprinting the age and sex dependency of the expression of cytochrome P-450_{6β} (gene IIIA2) and is also required for the maintenance of cytochrome P-450 PB_{2a} (gene IIIA1) during adult life.

Cytochrome P-450_{6β} (gene IIIA2) was induced in both sexes by PCN and dexamethasone (Heuman et al 1982, Scheutz et al 1984) and by phenobarbital (Heuman et al 1982, Guengerich et al 1982, Waxman 1988). The enzyme was reported to be increased >70-fold in female rats upon phenobarbital administration (Waxman et al 1985), thereby abolishing the sex differences in 6β-hydroxylation between male and female animals.

Waxman et al (1988b) have recently reported that 6β-hydroxylation is the major pathway of metabolism of testosterone, androstenedione and progesterone in humans, as compared to the multiple hydroxylative pathways that exist in the rat. Antibodies to cytochrome P-450_{NF} (gene IIIA4) and TAO (characteristic of gene subfamily IIIA) established that cytochrome P-450_{NF} is the major 6β-hydroxylase in human liver (Guengerich et al 1986), and is 75% similar to rat cytochrome P-450_{PCN} (gene IIIA1) (Gonzalez et al 1985).

1.4.4 CYTOCHROME P-450-INDEPENDENT STEROID METABOLISM

a) 5 α -reductase

5 α -reductase is a microsomal membrane-bound enzyme, and although it does not contain cytochrome P-450, it is thought to act in a similar manner to the monooxygenase system and consists of coenzyme Q₁₀ and NADPH-cytochrome oxidoreductase (Golf and Graef 1978). Thus electrons are transferred from NADPH to coenzyme Q₁₀ by NADPH-cytochrome oxidoreductase, and from coenzyme Q₁₀ to substrate by 5 α -reductase.

5 α -reductase activity has been reported to be higher in female rats (Yates et al 1958, Gustafsson and Stenberg 1976), and its developmental profile showed that 5 α -reductase activity was induced at puberty in female rats (Waxman et al 1985).

The hepatic expression of 5 α -reductase activity was increased upon exposure of male rats to oestrogens (Berg and Gustafsson 1973), but the expression of this female-specific enzyme was only partially affected by ovariectomy or oestrogen treatment of female rats. 5 α -reductase activity was fully suppressed upon postpubertal androgen exposure (Dannan et al 1986, Rumbaugh and Colby 1980), whilst castration increased 5 α -reductase activity (Yates et al 1958, Berg and Gustafsson 1973).

Hypophysectomy reduced 5 α -reductase activity markedly in female rats (Miller and Colàs 1982, Colby et al 1974, Kramer and Colby 1976), and was restored upon continuous GH administration (Mode et al 1981). Continuous GH infusion of male rats also led to the expression of this female-specific

enzyme (Mode et al 1980). Thus the increase in 5 α -reductase activity in female rats correlates well with the continuous secretory pattern of GH at puberty, showing that 5 α -reductase activity is regulated by the GH secretory pattern. The suppression of 5 α -reductase activity by testosterone therefore explains the relative absence of this enzyme in male animals (Dannan et al 1986).

b) 17-oxosteroid oxidoreductase

17 α /17 β -oxosteroid oxidoreductase and 3 α /3 β -oxosteroid oxidoreductase (Penning et al 1987) are also cytochrome P-450-independent microsomal enzymes. The mechanism of action of these enzymes is not clear as yet, although a mechanism for testicular 17 β -oxosteroid oxidoreductase (Inano and Tamaoki 1986) and 17 β -oxosteroid oxidoreductase from rabbit liver (Antoun et al 1985) has been discussed.

1.5 GROWTH HORMONE

1.5.1 STRUCTURE AND VARIANTS OF GROWTH HORMONE

Growth hormone (GH), in all species studied, is now known to be a protein of molecular weight 21kDa containing a single polypeptide chain of about 200 amino acids. Li et al (1966) first determined the complete amino acid sequence of growth hormone, although errors have subsequently been noticed using molecular biology techniques. The primary structure of the growth hormone molecule contains two disulphide bridges, one of which links distant parts of the

molecule whilst the other forms a small loop near the C-terminus. In addition, 50-60% of the growth hormone polypeptide chain exists in the α -helical conformation (Moore et al 1980).

A considerable amount of variation in the amino acid sequence of growth hormone has been determined between different species (Wallis 1981), resulting in differences in physicochemical properties. For instance, bovine growth hormone (bGH) is a fairly basic protein ($pI=8$), whereas human growth hormone (hGH) is acidic ($pI=5$). There is also virtually no immunological cross-reaction between hGH and bGH.

The 20K variant of hGH, which has a single chain that is 15 residues shorter than the normal 22K form of hGH, has been the subject of a recent review (Lewis et al 1987). The 20K variant accounts for 10-15% of pituitary hGH and lacks residues 32 to 46 as compared to 22K hGH. While the 20K variant retains full growth-promoting and diabetogenic activity (Kostyo et al 1985), and stimulates somatomedin production, it binds to growth hormone receptors with a lower affinity as compared to 22K hGH.

Mondon et al (1988) have recently demonstrated that a peptide representing this deleted region in 20K hGH (residues 32-46) may be responsible for the insulin-like activity of growth hormone, and therefore may explain previous reports that 20K hGH had impaired insulin-like effects. Salem (1988) has also reported that the effects of a naturally occurring pituitary peptide, hGH (1-43) potentiated the effects of insulin in normal, diabetic and

hypophysectomised rats.

1.5.2 SYNTHESIS AND SECRETION OF GROWTH HORMONE

1.5.2.1 BIOSYNTHESIS

Growth hormone is synthesised and secreted in the pituitary by a group of acidophilic cells termed somatotrophs (Bancroft et al 1976). In male animals, these are the predominant cells of the pituitary, whereas somatotrophs and lactotrophs (secrete prolactin) exist in equal numbers in female rats. Within the somatotroph, growth hormone is stored in the form of 300-400nm diameter granules prior to secretion. Growth hormone is derived from a larger precursor peptide, pre-growth hormone, of molecular weight 28kDa, first documented by Sussman et al in 1976. The amino acid sequence of preGH has been determined, and shown to be a 26 amino acid extension of the growth hormone molecule at the N-terminus, as has been observed with insulin.

1.5.2.2 SECRETION

It is now widely accepted that growth hormone secretion is episodic in nature. Edén (1979) demonstrated that the pattern of growth hormone secretion in adult rats was sexually differentiated, in that adult males exhibited an irregular growth hormone secretory profile, whereas growth hormone was secreted in a more continuous manner in adult females. Mode et al (1982) have shown that the secretory

pattern of growth hormone was an important determinant of the sex differences observed in hepatic steroid metabolism, and on the effects on body growth (Jansson et al 1985, Clark et al 1985, Isaksson et al 1978, Norstedt and Palmiter 1984).

No apparent sex differences have been reported in the secretory pattern of growth hormone secretion in humans. Albertsson-Wikland and Rosberg (1988) recently showed that although growth hormone secretion correlated well with height, there was no correlation between growth hormone secretion and the age and sex of children.

The human growth curve can, however, be divided into three distinct phases. There is a period of rapid and rapidly decelerating growth during infancy, followed by a period of steady and slowly decelerating growth during childhood. Growth appears to be completed by the adolescent growth spurt which, due to its greater magnitude in males than females, results in the sex differences in adult height (Brook et al 1988).

Normal adults secrete approximately 44µg growth hormone daily, whereas young adults secrete about 700µg. The basal growth hormone levels in man are about 2ng/ml, and increase episodically to about 10-30ng/ml. The bulk of growth hormone secretion in children occurs during sleep, especially during the first hour of onset (Sassin et al 1972).

Growth hormone in the circulation is now thought to be associated with a binding protein (Baumann et al 1988a and b), and a considerable amount of growth hormone circulates as a dimer in man.

The secretion of growth hormone is regulated and maintained by a balance between two hypothalamic hormones, growth hormone-releasing hormone and somatostatin.

1.5.2.3 GROWTH HORMONE RELEASING HORMONE (GHRH)

Although the presence of GHRH has been postulated for several years now, it was not isolated and characterised until 1982. Two groups independently showed that peptides with growth hormone-releasing activity, isolated from patients with pancreatic tumours, had given rise to acromegaly due to excessive growth hormone secretion (Guillemin et al 1982, Vale et al 1983). Guillemin et al (1982) isolated a peptide comprising of 44 amino acids and an amidated C-terminus, whereas Vale et al (1983) isolated GHRH containing 40 amino acids and a free C-terminus. Both molecules appeared to be equipotent in their function.

The precursor of GHRH has also been identified and is considerably larger than the GHRH molecule. Proteolytic cleavage of this molecule is thought to release other biologically active peptides in addition to GHRH (Gubler et al 1983).

1.5.2.4 SOMATOSTATIN

Somatostatin was first isolated and characterised by Brazeau et al in 1973 during the search for GHRH. This growth hormone release-inhibitory factor was shown to be a peptide consisting of 14 amino acid residues, and has been

isolated from the pancreatic D cells and the gut, in addition to the hypothalamus. Somatostatin appears to be synthesised in the form of a precursor protein containing about 120 amino acids with the somatostatin sequence at the C-terminus (Shen et al 1982).

Somatostatin has also been shown to inhibit the secretions of thyrotrophin, insulin, glucagon, pancreatic enzymes, gastric acid and other gut hormones, as well as growth hormone (Brazeau et al 1978). Tannenbaum et al (1978) assigned a physiological role to somatostatin exclusively as a growth hormone release-inhibitory hormone by the use of anti-somatostatin.

1.5.2.5 CONTROL OF GROWTH HORMONE SECRETION

The episodic release of growth hormone was shown to be principally controlled by an interaction between GHRH (Plotsky and Vale 1985) and somatostatin (Tannenbaum 1985, McIntosh et al 1988). Clark et al (1987) have demonstrated that whereas the underlying pattern of GHRH secretion was the same in rats, the somatostatin secretory pattern varied, resulting in the characteristic secretory profiles of growth hormone in male and female rats. In contrast, other authors have previously shown that GH secretion predominantly reflects the pulsatile release of GHRH, and that the trough levels of GH are mainly influenced by somatostatin (Tannenbaum et al 1983, Wehrenberg et al 1982, Sato et al 1988). Further, GH pulses may also be accompanied by reduced somatostatin secretion (Tannenbaum and Ling 1984, Plotsky

and Vale 1985).

In addition it has been suggested that growth hormone can regulate its own secretion by a negative feedback mechanism. Krulich and McCann (1986) first demonstrated that bGH administration into rats reduced pituitary growth hormone content, and this feedback inhibition has subsequently been confirmed by other authors (Groesbeck et al 1987, Willoughby et al 1980, Tannenbaum 1980).

It has further been shown that growth hormone does not inhibit its secretion at the pituitary level (Richman et al 1981, Goodyer et al 1984), and may act at the level of the hypothalamus to either increase somatostatin secretion and/or decrease GHRH release. Certainly, evidence exists that growth hormone increases somatostatin release (Chihara et al 1981), and that somatostatin inhibits growth hormone release in rats (Brazeau et al 1974, Tannenbaum 1985). Clark et al (1988) have more recently confirmed that growth hormone inhibits its own release in conscious rats, and further showed that the feedback effects of growth hormone involve an inhibition of GHRH release rather than an increase in somatostatin secretion, and take place at the level of the hypothalamus.

While GHRH is thought to act via both cyclic AMP- (Ray and Wallis 1988, Dana and Karin 1989) and protein kinase C-dependent (Ohmura et al 1988, French et al 1989) mechanisms, somatostatin is thought to alter calcium levels in somatotrophs (Holl et al 1988). The regulation of GH secretion has recently been reviewed by Thorner (1989).

Noguchi et al (1989) have suggested that IGF-I may

regulate growth hormone feedback at the level of the pituitary and hypothalamus since a substance that cross-reacted with IGF-I was detected in bovine anterior pituitary cells.

Glucocorticoids are reported to increase, and thyroid hormones to decrease growth hormone secretion by adult human (Bridson and Kohler 1970, Mulchahey et al 1988) and rat (Oosterom et al 1983) pituitary cells, although the effects of glucocorticoids are documented to be inhibitory in vivo (Nakagawa et al 1973). The effects of the gonadal hormones on growth hormone secretion were also reported to be opposite in vivo and in vitro (Dieguez et al 1988). Nakagawa et al (1987) have reported that the suppressing effect of glucocorticoids on growth hormone release in vivo was at least partially due to the increase in hypothalamic somatostatin release, together with a decrease in GHRH release, and that these effects probably overwhelmed the potentiating effect of glucocorticoids on growth hormone release at the pituitary level, thereby resulting in a net inhibitory effect in vivo.

These observations are further supported by the finding that glucocorticoids increased gene transcription (Tushinski et al 1977) and that the activated glucocorticoid receptor protein binds to the first intron of the hGH gene (Moore et al 1985).

Growth hormone synthesis and secretion also appears to be dependent on thyroid hormones, which mediate their effects by regulation of the transcriptional rate of the growth hormone gene (Dieguez et al 1988).

However, it has recently been suggested that both thyroid hormones and glucocorticoids influence GH secretion by affecting the responsiveness of the somatotroph to GHRH (Edwards et al 1989). In addition, the secretion of GH was impaired in animals with a deficiency of androgen receptors. Thus GH secretion was highest in females, lowest in males and intermediate in testicular-feminised rats, therefore indicating a partial masculinisation response as compared to normal animals (Batson et al 1989).

Growth hormone secretion is also regulated by a certain degree of metabolic control. Glucose administration lowered growth hormone secretion in normal subjects, whereas hypoglycemia is known to stimulate growth hormone release.

A protein-rich meal or infusion of amino acids is also reported to cause growth hormone release. Paradoxically, protein deficient states also result in growth hormone release. Fatty acids suppressed growth hormone responses to certain stimuli, including amino acid infusion and hypoglycemia. Fasting is documented to stimulate growth hormone secretion, possibly as a means of mobilising fat as an energy source and preventing protein breakdown (Holly et al 1988).

The neural control of growth hormone secretion has been reviewed recently by Dieguez et al (1988).

1.5.3 GROWTH HORMONE RECEPTORS

Studies on the growth hormone receptor have recently been reviewed by Hughes and Friesen (1985) and Roupas and

Herington (1989).

Growth hormone receptors have been characterised in a number of tissues (Hughes and Friesen 1985), although the most widely studied tissue has been the liver (Ranke et al 1976) since growth hormone has many actions on hepatic tissue. In addition, many of the actions of growth hormone are mediated by the somatomedins which are produced by the liver. The liver also contains lactogenic receptors for prolactin (Posner 1976), and much confusion has arisen in the past owing to the lactogenic and somatogenic properties of human GH (hGH). In contrast, rat growth hormone (rGH) and bovine growth hormone (bGH) specifically bind to somatogenic receptors, and rat prolactin (rPRL) to lactogenic receptors.

GH binding sites have been identified in male rat hepatocytes (Ranke et al 1976), and the covalent binding of radiolabelled GH to these binding sites studied (Donner 1983, Yamada and Donner 1984). Affinity chromatography studies identified the GH receptor as a 220,000 daltons molecule (Waters and Friesen 1979). The growth hormone receptor from rabbit liver appears to have a subunit molecular weight of 50,000-80,000 daltons on SDS-PAGE, whereas that from rat and human liver has been estimated at 100,000-110,000 daltons. Leung et al (1987) have recently cloned (Wallis 1987) and sequenced the rabbit growth hormone receptor and reported its molecular weight to be 130,000 daltons.

Biochemical characterisation of the growth hormone receptor has shown that it contains carbohydrate residues (binds to concanavalin A), but does not contain sialic acid

residues (unaffected by neuraminidase). In addition, galactose residues may be required for receptor activity, as shown by abolition of receptor responsiveness after treatment with galactosidase. Studies with phospholipase C revealed that phospholipids are not of any importance to the growth hormone receptor (Hughes and Friesen 1985).

Disulphide bonds in the membrane appear to be intimately involved in the maintenance of a receptor structure necessary for growth hormone binding. In addition, disruption of the disulphide bonds resulted in increased dissociation and displacement of bound hormone, indicating a possible role in the irreversible nature of growth hormone binding, which may be the irreversible component previously identified in hormone-receptor interactions (Moore et al 1983).

There is now evidence for the existence of a heterogenous population of growth hormone receptors in rabbit liver (Hughes and Friesen 1985). It has been proposed that three receptor populations existed in this tissue :

i) GHR-1 (1%) binds rat, rabbit and human growth hormone and the 20K variant of human growth hormone.

ii) GHR-2 (85%-90%) binds human growth hormone with high affinity and other forms with low affinity.

iii) PRLR (10-15%) binds both 20K and 22K human growth hormone, as well as prolactin.

Different GH-receptor populations are involved in the insulin-like and lipolytic actions of GH in adipocytes (Chipkin et al 1989), which is supported by previous reports that higher GH concentrations are required for the

insulin-like effect of GH.

Male hepatocytes bind approximately 2000 hormone molecules per cell with a dissociation constant of $3 \times 10^{-10} \text{M}$. Female rat hepatocytes bind 5-10 times more growth hormone at the same concentration (Postel-Vinay and Desbuquois 1977).

A large proportion of the receptors in rabbit liver are membrane-bound. Although receptors associated with the plasma membrane are probably involved with the initiation of the growth hormone response, receptors on intracellular membranes (eg endoplasmic reticulum) do not appear to have an obvious role. These may represent either newly synthesised or spare receptors, or receptors that have been recently internalised. These cytosolic growth hormone receptors have identical binding characteristics to those of the plasma membrane. In addition, a cytosolic binding protein has recently been identified and is thought to be a cleaved form of the growth hormone receptor. Baumann et al (1988) have recently reported that a substantial fraction of circulating hGH in man circulates in the form of a growth hormone-binding protein complex. The binding protein has a native molecular weight of approximately 100kDa and a binding subunit of about 60kDa (Hughes and Friesen 1985). The GH binding protein has recently been cloned and sequenced (Leung et al 1987) as a 51kDa molecule. In addition, it has recently been demonstrated that the growth hormone receptor from rabbit liver and the binding protein from rabbit serum have the same N-terminal amino acid sequence, indicating that the binding protein corresponds to

the extracellular hormone-binding domain of the liver receptor (Leung et al 1987). This finding therefore confirmed the suggestion that the serum growth hormone binding protein may be derived from the membrane-bound growth hormone receptor (Daughaday et al 1987). Measurement of the growth hormone binding protein levels in serum has been proposed as a noninvasive tool to study the ontogeny of growth hormone receptors in humans (Daughaday et al 1987).

The regulatory factors affecting growth hormone receptors have been extensively documented in a recent review (Roupas and Herington 1987).

Both hGH and rGH were capable of inducing PRL and GH receptors in hypophysectomised male and female rats (Mode et al 1983, Baxter and Zaltsman 1984), although rPRL did not induce GH receptors (Baxter et al 1984).

Baxter et al (1980) have suggested that GH receptor number is either directly or indirectly dependent on the presence of GH, oestradiol and insulin. Hepatic GH receptors were decreased in diabetic rats, as was serum GH (Scott and Baxter 1986). Hepatic GH-binding was also decreased in STZ-diabetic rats (Baxter et al 1980). The partial restoration of this effect by insulin indicated a regulatory role of insulin in the control of GH binding (Baxter and Turtle 1978).

Hypophysectomy has been shown to increase liver GH-binding sites (Picard and Postel-Vinay 1984), an effect that was reversible upon GH administration (Posner 1984). Maes et al (1983a) have also reported a decrease in circulating GH concentrations in hypophysectomised rats,

which correlated well with an increase in GH receptor number.

Baxter and Zaltsman (1984), however, reported a sex-differentiated effect of hypophysectomy on GH receptors. Whereas hypophysectomy reduced the number of GH receptors in females, receptor content was increased in male rats. This discrepancy in receptor content upon removal of the GH influence may be related to the sex difference in the GH secretory pattern (Edén 1979).

Intermittent growth hormone administration (Maiter et al 1988a), mimicking the male secretory pattern, resulted in greater increases in body weight and serum somatomedin levels than continuous growth hormone infusion. As would be expected, continuous growth hormone infusion increased growth hormone receptor number markedly whereas intermittent administration had no effects. This would suggest that a continuous presence of growth hormone is required for the upregulation of its receptors. Further, only continuous growth hormone infusion stimulated prolactin receptor content, indicating that the regulation of growth hormone and prolactin receptors may be linked (Norstedt 1982, Norstedt et al 1981). In a subsequent study, Maiter et al (1988b) demonstrated that whereas the administration of growth hormone over short time periods led to a time- and dose-dependent down-regulation of its receptors, stimulatory effects were observed upon prolonged exposure (Baxter et al 1984, Baxter and Zaltsman 1984), which is consistent with ligand-induced internalisation and degradation of the receptor.

Bullier-Picard et al (1989) reported that ^{125}I -labelled hGH was internalised by male rat hepatocytes with a sequential association with plasma membranes and endocytotic structures distinct from golgi elements, and degraded in lysosomes (Wakai et al 1984). In addition, growth hormone is thought to be internalised by two endocytotic compartments (Husman et al 1988) prior to lysosomal degradation.

Insulin-like growth factor 1 (IGF-I) levels are low in rats at birth (Sara et al 1980) despite high growth hormone levels (Ojeda and Jameson 1977). At puberty there is a progressive rise in these growth factor levels (Edén 1979) associated with increased growth and growth hormone secretion. The discrepancy at birth and correlation during puberty between somatomedin and growth hormone levels suggest ontogenic variations in the responsiveness of the liver to growth hormone.

Maes et al (1983a) have reported on the development of growth hormone receptors in male and female rats and showed that there was a rise in receptor number before puberty in male and female rats (Postel-Vinay and Desbuquois 1977), which preceded the increase in growth velocity and plasma growth hormone levels by about one week. Whereas growth hormone receptor concentration continued to increase until 120 days in females, male rat livers showed a significant decrease by day 50. Similarly, prolactin receptor content remained constant in male rat liver but increased in females, and with an inverse relationship to plasma testosterone levels. Pregnancy also caused marked changes in GH binding (Postel-Vinay and Desbuquois 1977).

Contrary to the induction of GH receptors upon GH administration to rats (Baxter and Zaltsman 1984), a decrease in receptor number has been reported in human IM-9 lymphocytes exposed to GH (Lesniak and Roth 1976). This down-regulation of GH receptors by GH that is observed in humans (Palmer and Wallis 1989) has not yet been demonstrated in rats, not even upon administration of high doses of GH to rats bearing pituitary tumours (Baxter et al 1982).

1.5.4 POST-RECEPTOR ACTIONS OF GROWTH HORMONE

Very little is known about the mechanism of growth hormone action (reviewed by Isaksson et al 1985). Although it is well established that many of the effects of growth hormone, particularly on skeletal muscle, are mediated by the somatomedins (see section 1.5.5), the direct effects following stimulation of growth hormone receptors are relatively unknown.

Many direct effects of growth hormone on protein synthesis and lipid metabolism, DNA synthesis and enzyme activity have been reported, but these are all late events. It is now well known that the effects of growth hormone in the diaphragm are mediated by increased amino acid incorporation and protein synthesis, and due to an increase in RNA transcription in the perfused rat liver (Kostyo 1968, Kostyo and Nutting 1973, Rillema and Kostyo 1971). This stimulation of protein synthesis by growth hormone is thought to be due to its insulin-like action (Cameron et al

1988). The effects of growth hormone on carbohydrate and lipid metabolism have recently been reviewed by Davidson (1987). In general, growth hormone shows anti-insulin-like effects in the long term and insulin-like effects over shorter periods, as has been confirmed both in vivo and in vitro (Betley et al 1989).

Attempts to link growth hormone receptor stimulation with adenylate cyclase in response to GH have given conflicting results. Some studies have demonstrated the inhibition of adenylate cyclase (Albertsson-Wikland and Rosberg 1982) while others failed to show this effect (Hughes and Friesen 1985). Leppert et al (1981) showed that growth hormone restored glucagon-stimulated increases in adenylate cyclase activity in hypophysectomised rats. In addition, the increased adenylate cyclase activity was reported to reside in the catalytic subunit (Hung and Moore 1984). Several studies have, however, reported an indirect effect of adenylate cyclase in growth hormone action represented by alterations in intracellular cyclic AMP levels.

Cyclic AMP levels were unchanged by hypophysectomy of male rats (Isaksson et al 1974). In addition, administration of growth hormone to these hypophysectomised animals elicited no change in intracellular cyclic AMP levels, showing that cyclic AMP was not involved (Isaksson et al 1974, Klingensmith et al 1980). In contrast, Thompson et al (1973) demonstrated that hGH administration to hypophysectomised rats resulted in the activation of cyclic AMP phosphodiesterase, thus implying a role of the cyclic

AMP system in the mechanism of action of growth hormone. Similarly, cyclic AMP-dependent protein kinase activity was stimulated by GH (Byus et al 1978).

The lipolytic action of growth hormone in adipocytes (reviewed by Goodman et al 1987, Schaffer 1985) was reported to involve both protein synthesis and an accumulation of cyclic AMP (Moskowitz and Fain 1970, Fain et al 1971). Growth hormone was also reported to stimulate both lipolysis and cyclic AMP levels in perfused fat cells (Sengupta et al 1981, Solomon et al 1987). The ability of theophylline, which inhibits cyclic AMP phosphodiesterase, to abolish the effects of growth hormone on amino acid transport and protein synthesis suggested that growth hormone may be acting by decreasing cyclic AMP levels (Payne and Kostyo 1970, Rillema et al 1973).

A recent report showed that physiological concentrations of growth hormone enhanced guanylate cyclase and cyclic GMP systems in vitro (Vesely 1981), and may therefore be a possible mechanism of action of growth hormone. Guanylate cyclase, however, was not activated by growth hormone in the diaphragm (Ahrén et al 1976).

Grief et al (1982) demonstrated that both thyroxine and growth hormone stimulated the efflux of calcium from rat liver mitochondria.

Smal et al (1989), using an inhibitor of protein kinase C known as acridine orange, demonstrated that growth hormone stimulated lipogenesis in rat adipocytes by protein kinase C activation. It has further been proposed that growth hormone stimulated protein kinase C by means of diacylglycerol

formation (Doglio et al 1989).

Some evidence exists that the activated growth hormone receptor may act as a protein kinase, but again this data has been conflicted. Binding of growth hormone to rat hepatocytes led to a rapid (<10minutes) phosphorylation of several cellular proteins, including one of molecular weight 46kDa (Yamada et al 1987).

Thus there appears to be no overall consensus on the intracellular mediator of growth hormone action.

1.5.5 INSULIN-LIKE GROWTH FACTORS

Salmon and Daughaday (1957) first proposed that the anabolic effects of growth hormone on cartilage were mediated by a putative sulphation factor, now known as somatomedin (Daughaday et al 1972). It is now evident that a whole family of somatomedins exist. IGF-I (molecular weight 8000-9000 kDa) is a single chain polypeptide containing 70 amino acid residues and three intrachain disulphide bridges, and is identical to somatomedin C (Klapper et al 1983). IGF-II is thought to be identical to multiple stimulating activity (MSA). Both insulin-like growth factors (IGF) I and II are reported to show marked amino acid sequence homology to insulin. In addition, the somatomedins mimic many of the metabolic effects of insulin (Froesch et al 1985, Zapf et al 1981).

The somatomedins are produced in the form of a large precursor molecule by, but not stored in, the liver, kidney and several other tissues (Hintz 1987). The perfused liver

has been a commonly used tissue for the study of IGF-1 as it is its major site of synthesis (Schalch et al 1979). Although there is no evidence that somatomedins act by an endocrine mechanism (ie synthesised by the liver and released into the general circulation), an autocrine or paracrine mechanism of action has been demonstrated (D'Ercole et al 1984, Holly and Wass 1989). This is supported by the observation that growth hormone stimulated growth and tissue IGF-I levels without corresponding increases in circulating IGF-I levels, thereby implying that autocrine or paracrine IGF-I mediated the effects of growth hormone in young animals (Orlowski and Chernausek 1988).

Phillips et al (1976) reported a high molecular weight circulating form of IGF-I, which either represented the IGF-I precursor, or IGF-I bound to an additional molecule. Somatomedins are now known to circulate in association with high molecular weight specific binding proteins (Zapf et al 1975, Moses et al 1976, Moses et al 1979, Ooi and Herington 1988). At least two binding proteins have been detected with molecular weights of 40-60kDa and 150kDa (Kaufmann et al 1978, Moses et al 1976, Binoux et al 1982), although three categories of binding proteins have been documented according to their binding specificities (Forbes et al 1988). Growth hormone appears to stimulate the production of the 150K binding protein (Kaufmann et al 1978, Moses et al 1976), whereas the 40-60K binding protein appears to be regulated in a growth hormone-independent manner (Binoux et al 1982). At least six growth hormone-dependent binding proteins have been isolated (D'Ercole et al 1984). The

growth hormone-independent binding protein is particularly abundant during foetal life (D'Ercole et al 1980), in hypophysectomised animals (Hintz et al 1981) and in growth hormone-deficient patients (D'Ercole et al 1984). Their function, as yet, appears to be the extension of serum somatomedin half-life (Smith 1984, D'Ercole et al 1984) which, in its free form is metabolised in about ten minutes (Cohen and Nissley 1976). In addition, the binding proteins also appear to modulate the biological activity of IGF-I (Zapf et al 1979) directly by supporting IGF-binding at the cell surface (Clemmons et al 1986, Barenton et al 1988).

Growth hormone administration is known to stimulate IGF-I production by the liver in vitro (Daughaday et al 1976, Binoux et al 1982, Scott et al 1985a & 1985b). Hepatic production of IGF-I in vivo correlates with serum IGF-I levels (Schwander et al 1983), which correlates well with IGF-I mRNA levels. It therefore appears that the metabolic regulation of IGF-I is at the mRNA level (Goldstein et al 1988). Further, Norstedt and Möller (1987) showed that growth hormone could directly induce IGF-I mRNA in hepatocyte cultures.

It is now well established that the effects of growth hormone on skeletal growth are mediated by IGF-I (Froesch et al 1985). However, it is not yet fully clear as to which of the other effects of growth hormone are direct and which are mediated by somatomedins.

IGF-I levels are lower in hypophysectomised than normal rats (Scott et al 1985a;b, Miller et al 1981). Infusion of growth hormone to hypophysectomised rats in a intermittent

manner resulted in greater increases in serum IGF-I levels and IGF-I mRNA levels than continuous administration (Isgaard et al 1988), thus confirming the observations of previous studies (Jansson et al 1982, Clark et al 1985). In addition, both local (Phillips et al 1980) and hepatic IGF-I (Isaksson et al 1982, Isgaard et al 1986) are reported to stimulate growth in hypophysectomised rats (Schoenle et al 1982). Conversely, IGF-I expression was increased in transgenic mice (GH fusion genes) (Mathews et al 1988) and tumour-bearing rats (Scott et al 1985) together with elevated growth hormone levels, which correlated with increased IGF-I levels and increased growth.

Insulin deprivation also interferes with growth hormone-dependent IGF-I synthesis and secretion (reviewed by Holly et al 1988). IGF-I levels were reduced in diabetic rats, and could be accounted for either directly by reduced IGF synthesis or indirectly by reduced GH secretion or receptor content (Scott and Baxter 1986). Plasma IGF-I levels are reported to be reduced in diabetes (Maes et al 1983b), possibly as a result of impaired synthesis by the liver (Miller et al 1981). Since IGF-I levels in diabetics are not restored by treatment with growth hormone, it was suggested that the diabetic liver may be resistant to growth hormone (Phillips et al 1976), possibly due to a reduction in growth hormone receptors (Scott and Baxter 1986). In contrast, Maes et al (1986) found no changes in receptor content in diabetes and suggested that the low serum somatomedin levels were due to a defect distal to binding of growth hormone to its receptor. In rats made mildly diabetic

with streptozotocin, insulin restored serum IGF-I levels, as well as growth parameters in the diabetic animals (Maes et al 1983, Taylor et al 1987). This reversal was, however, only observed when insulin was administered via the hepatic portal vein, and not the jugular vein, indicating that the liver was the target organ in mediating the growth-promoting effects of insulin (Griffen et al 1987). This is consistent with previous observations that insulin stimulates IGF-I production by the isolated perfused liver (Kogawa et al 1982) and cultured hepatocytes (Binoux et al 1982, Kogawa et al 1983, Scott and Baxter 1986). In addition, insulin also stimulated the accumulation of IGF-I mRNA transcripts in cultured hepatocytes (Johnson et al 1989).

The mechanism by which growth hormone stimulates IGF-I production is unknown, but it is clear that an action of GH on the expression of the IGF-I gene is involved, since growth hormone specifically increases IGF-I mRNA transcription in liver (Mathews et al 1986, Johnson et al 1989) and adipose cells (Doglio et al 1987). Further, Scott et al (1985a) showed that although IGF-I production was inhibited by cycloheximide, actinomycin D was less effective, thereby suggesting that continuous protein synthesis is required (Murphy and Luo 1989) and that a stable species of mRNA was present. In addition, the increase in expression of IGF-1 in response to growth hormone was suggested to be mediated by phospholipase C (Doglio et al 1989).

IGF-I receptors show structural similarity to the insulin receptor (section 1.8.1) (Rechler et al 1980,

Chernausek et al 1981, Massagué and Czech 1982, Watanabe et al 1987) and are thought to contain two extracellular α and two transmembrane β polypeptide chains joined by disulphide bridges, and also appear to bind insulin quite effectively. In addition, the α -subunit contains the IGF-I binding site and a cysteine-rich region, whereas the β -subunit contains a ligand-activated tyrosine kinase domain and autophosphorylation sites. The IGF-I receptor, in analogy with the insulin receptor, is associated with a protein kinase which is activated upon binding to IGF-I, and leads to phosphorylation of tyrosine residues of cellular proteins. The human IGF-I receptor has also been characterised (Steele-Perkins et al 1988). The IGF-II receptor appears to only contain a single transmembrane polypeptide chain (Morgan et al 1987) and does not bind to insulin.

1.6 MECHANISM OF ACTION OF GLUCOCORTICOIDS

Glucocorticoid action is initiated by entry of the steroid into the cell and binding to the cytosolic glucocorticoid receptor proteins. The resulting steroid hormone receptor complexes then undergo activation (or transformation) and are translocated to the nucleus where they interact with nuclear chromatin acceptor sites (Pfahl 1986). The phosphorylation of steroid hormone receptors has also been implicated in the interaction between hormone-receptor complexes and DNA components (Auricchio 1989). The activated glucocorticoid receptor appears to have a high affinity for the arginine-rich histones 3 and 4 (Ueda et al 1989). Activated hormone-receptor complexes then bind to DNA regulatory sequences, glucocorticoid responsive elements (GRE) (reviewed by Beato et al 1989), of specific genes leading to the specific transcription and expression of these genes. Both human and rat growth hormone genes have a GRE in the structural gene, and are therefore glucocorticoid-responsive (Perry et al 1988).

Hormone-receptor complexes are known to be enhancers of transcription. The resulting complexes effect the glucocorticoid response, which may be inhibitory or stimulatory, depending on the system involved. For instance, dexamethasone stimulated tyrosine aminotransferase activity but reduced the expression of HMG-CoA reductase mRNA, and adrenalectomy increased its expression (Simonet and Ness 1989).

Although the passage of steroids through the plasma

membrane has been assumed to occur by simple diffusion due to their lipophilic nature, several authors have proposed the presence of a glucocorticoid binding-site at the plasma membrane that is responsible for the transport of steroids into the cell cytoplasm (Plas and Duval 1986). Howell et al (1989) have recently identified a 45K dexamethasone-binding site on the plasma membrane that may be responsible for the active transport of glucocorticoids into the cell.

Rousseau et al (1972) proposed that liver hepatoma cells contained a single class of cytoplasmic glucocorticoid receptors which were similar to the Type II corticosteroid receptors identified in the kidney. At least four forms of glucocorticoid receptors, the active and inactive steroid binding forms of the unoccupied receptor and the activated and unactivated forms of the occupied receptor, are now thought to exist (Harmon et al 1988). The proposed model of the structure of the glucocorticoid receptor has at least three functionally distinct domains (Carlstedt-Duke et al 1982) : a protein that binds glucocorticoid, a region that binds to DNA, and a modulating (immunoreactive) domain whose function is not clear as yet. Sequencing and expression of cDNA clones of the glucocorticoid receptor have revealed that the DNA-binding region is rich in cysteine, arginine and lysine residues, and contains a cys-X₂-cys region that probably forms metal-binding fingers. Such fingers are well suited for binding specific sequences in DNA. Mutants of the glucocorticoid receptor containing insertions that disrupt the cys-X₂-cys region bind hormone, but do not activate transcription, showing that this region is important for

glucocorticoid action.

Hydrocortisone administration to male rats caused a down-regulation of the glucocorticoid receptor which correlated well with a decrease in tyrosine aminotransferase activity, a characteristic glucocorticoid function (Yi-Li et al 1989). A similar effect has been reported in mice (Svec 1988).

Permissive hormones such as the glucocorticoids have been reported to modulate the actions of hormones that act through cyclic AMP (Malbon et al 1988, Hems and Whitton 1980), and adenylate cyclase (Davies and Lefkowitz 1984). Further, adrenalectomy affected the steady-state amounts of the components of the hormone-sensitive adenylate cyclase system : a 30% decrease in the amount of the α -subunit of G_s and a 50% decrease in the amount of the β -subunit of G_s (Ros et al 1989) was observed, an effect that was reversed upon dexamethasone replacement therapy. However, adenylate cyclase activity was markedly reduced and β -receptor expression increased with the same treatment (Ros et al 1989).

The expression of the β -adrenergic receptor, which is coupled to adenylate cyclase via guanine-nucleotide binding proteins (G-proteins), is stimulated by glucocorticoids (Graziano and Gilman 1987), whereas receptor sensitivity is reduced by adrenalectomy or depletion of adrenal steroids (Davies et al 1981). The regulation of G-protein-linked receptors by these hormones remains obscure. The gene for the mammalian β -adrenergic receptor has recently been isolated (Dixon et al 1986) and glucocorticoid responsive

elements (GRE), which are DNA sequences that mediate transcriptional regulation by glucocorticoids, have been identified in the 5'-coding (Kobilka et al 1987), coding and 3'-noncoding regions of this gene (Chung et al 1987). Further, the GREs in the 5'-noncoding region of the hamster β_2 -adrenergic receptor appear to be responsible for the stimulation of its mRNA and expression by glucocorticoids (Malbon and Hadcock 1988). Actinomycin D, a potent inhibitor of transcription, abolished the dexamethasone-induced increase in β -adrenergic receptor expression, showing that dexamethasone exerted its effects by influencing gene transcription (Hadcock and Malbon 1988).

Corticosteroids also interact directly with the GH gene to stimulate transcription (Evans et al 1982, Spindler et al 1982, Yaffe and Samuels 1984, Diamond and Goodman 1985, Gertz et al 1987).

The diabetic state is characterised by a fall in serum somatomedin levels and increased levels of glucocorticoids, it was recently reported that both adrenalectomised-diabetic and adrenalectomised-non-diabetic rats displayed decreases in serum somatomedin levels irrespective of glucocorticoid status (Hofert et al 1989). Conversely, Levinovitz and Norstedt (1989) reported that glucocorticoids play an important role in the expression of insulin-like growth factor II (IGF-II), since dexamethasone administration specifically and irreversibly reduced IGF-II expression in newborn rats.

1.7 MECHANISM OF ACTION OF THYROID HORMONES

The unbound thyroid hormones (triiodothyronine, T_3 and thyroxine, T_4) enter the cell by diffusion. However, increasing evidence is now accumulating to support the claim that transport of thyroid hormone into rat hepatocytes is an energy-dependent and saturable process (Hennemann et al 1986, Blondeau et al 1988, Docter et al 1988), rather than simple diffusion, since transport could be inhibited by specific monoclonal antibodies (Rao et al 1981). Internalised hormone then binds to cytosolic binding proteins (CBP), forming a hormone-CBP complex. The T_3 -CBP complex remains in a reversible equilibrium with a very small amount of free T_3 in the cytoplasm. T_4 is normally converted to T_3 before it acts in the nucleus. Unlike the steroid hormones, T_3 can directly interact with its nuclear receptor ($K_D = 10^{-9}M$), and cytoplasmic receptors are apparently not directly involved in the activation of the nuclear receptor.

The nuclear receptors are associated with chromatin, and are nonhistone proteins with a molecular weight of about 50K. The receptors can bind to DNA, which accounts for their presence in chromatin. Free T_3 does not combine with the DNA, so that the presence of these receptor proteins accounts for the observed association of T_3 with cell nuclei (Bernal and Refetoff 1977, Aprilletti and David-Inouye 1984).

Thyroid hormone receptors also appear to be present in the cytoplasm, inner mitochondrial membrane and plasma

membrane of the cell.

The physiological and developmental effects of the thyroid hormones are mediated by a family of thyroid hormone receptors. Three human thyroid hormone receptors have been characterised from skeletal muscle (Nakai et al 1988).

Thyroid hormone-receptor complexes function as trans-acting transcriptional factors and exert their effects by binding to specific DNA sequences in thyroid hormone-responsive genes (TRE). Messenger RNA formation and an increase in protein synthesis appear to follow receptor interaction (Bernal and Refetoff 1977).

The thyroid hormones, like the glucocorticoids, also exert permissive effects on the actions of hormones that act via cyclic AMP (Malbon et al 1988). It has been shown that thyroid hormones can differentially regulate the steady-state amounts of several G-protein subunits such as α -G_i (α -G₄₁), α -G_o (α -G₃₉) and β -G_{35/36} in adipocytes (Malbon et al 1985, Saggersen 1986, Milligan et al 1988, Ros et al 1989). In the liver, both thyroid status and adrenalectomy modulated the steady-state amounts of G-proteins (Stiles et al 1981, Garcia-Sainz et al 1988) and the β -adrenergic receptor (Wolfe et al 1976, Rajerison et al 1974).

Thyroid hormone responsive elements (TRE) have recently been identified on the growth hormone gene, and may account for the stimulation of this gene by thyroid hormones. The sequences mediating thyroid hormone stimulation are thought to be located in the 5'-flanking regions of the rGH gene (Ye and Samuels 1987), between -208 and -160 bp (Larson et al

1986, Brent et al 1989, Copp and Samuels 1989). Thus T_3 acts directly with the GH gene to stimulate transcription (Evans et al 1982, Spindler et al 1982, Yaffe and Samuels 1984, Diamond and Goodman 1985).

1.8 INSULIN

1.8.1 INSULIN RECEPTOR

1.8.1.1 RECEPTOR STRUCTURE

The insulin signal is initiated upon binding of insulin to its receptor. The structure of the insulin receptor has been identified by a multitude of techniques such as a) cross-linking of radiolabelled insulin to its receptor and then analysing the labelled proteins, b) receptor purification followed by sequencing of the isolated protein, and c) immunoprecipitation of the insulin receptor (reviewed by Jacobs and Cuatrecasas 1981, Goldfine 1987).

The purification of the insulin receptor has recently been documented extensively (Hughes and Friesen 1985). Based on these studies, a model for the insulin receptor has been proposed. The insulin receptor is a tetramer consisting of two 125-135K α - and two 95K β -subunits linked together by disulphide bonds (reviewed by Czech 1985). Thus the glycoprotein configuration is $(\beta-S-S-\alpha)-S-S-(\alpha-S-S-\beta)$, and contains two type I and one type II disulphide bonds.

The insulin binding site resides in the α -subunit of the receptor and is exposed to the extracellular

environment. Since no region of uncharged amino acids long enough to span the membrane has been identified in the α chain, it is unlikely to be transmembrane in nature (Yip et al 1988). The α -subunit has 15 potential asparagine-linked glycosylation sites and is rich in cysteine residues. In fact, the cysteine-rich region between residues 155 and 312 of the α chain could either constitute the insulin receptor binding site or may be involved in receptor-receptor cross-linking. Similar high-cysteine regions have also been identified in the IGF-I receptor.

The β -subunit is the transmembrane protein and is therefore exposed to both the extracellular and cytosolic surfaces of the cell. The β -subunit has 4 potential asparagine-linked glycosylation sites and 4 extracellular cysteine residues. The transmembrane domain is probably represented by a long sequence of hydrophobic amino acids.

Insulin binding to the α -subunit of its receptor is thought to induce a conformational change (Schenker and Kohanski 1988), which in turn activates an intrinsic tyrosine kinase in the β -subunit which autophosphorylates the receptor. Several lines of evidence have indicated that this tyrosine-specific phosphorylation is an obligatory step in insulin action. An additional requirement for autophosphorylation appears to be the association of the $\alpha\beta$ insulin receptor heterodimer into the $\alpha_2\beta_2$ heterotetrameric form (Wilden et al 1989, Kubar and van Obberghen 1989).

1.8.1.2 RECEPTOR BIOSYNTHESIS

Pulse chase experiments have led to the elucidation of receptor biosynthesis. The α - and β -subunits are derived from a common precursor of the insulin receptor and is synthesised on the endoplasmic reticulum as a 1370 amino acid 190K molecule (Forsayeth et al 1986). Asparagine residues are co-translationally N-glycosylated, yielding the high-mannose form of the receptor. Sugar moieties appear to play an important role in insulin binding since tunicamycin blocked both N-glycosylation and insulin-binding. The receptor is then transferred to the golgi where the mannose residues are replaced by other sugars such as glucosamine and fucose, forming the 210K receptor species. The 210K form is then slowly proteolytically split to form the α' (125K) and β' (83K) chains. immediately before insertion of the subunits into the plasma membrane, sialic acid residues are acquired, giving rise to the mature α (135K) and β (90K) subunits (Forsayeth et al 1986).

Cloning of the insulin receptor has further led to the characterisation of the insulin receptor. The insulin receptor gene codes for the entire α - and β -subunits of the receptor in the form of a single precursor molecule. At the start of the α -subunit there is a 27 amino acid N-terminal signal sequence sequence which allows the receptor to enter the endoplasmic reticulum, and is subsequently cleaved. Next a sequence for the 719 amino acid α -subunit follows, then a 4-amino acid proteolytic cleavage site (arg-lys-arg-arg), and finally a sequence specifying the 620 amino acid

β -subunit.

1.8.1.3 RECEPTOR INTERNALISATION

Upon binding of insulin to its membrane receptor, the hormone-receptor complex has been shown to migrate and congregate at coated pits in the membrane. These coated pits are plasma membrane invaginations lined with protein clathrin on their cytoplasmic side, which is responsible for mediating the internalisation of activated hormone-receptor complexes. On entry into the coated pit, vesicularisation occurs, leading to the formation of an endosome vesicle. From here, the receptor can either be degraded via the lysosomes or alternatively recycled to the plasma membrane by interaction with the golgi system (Jones et al 1988, McClain and Olefsky 1988). This is thought to be the major pathway undertaken by the insulin-receptor complex, and although insulin itself is degraded in the process, its receptor is recycled intact to the plasma membrane.

An insulin-degrading enzyme has been implicated in the intracellular degradation of insulin, and has recently been identified as a cytosolic enzyme (Akiyama et al 1988).

Insulin-stimulated internalised receptors remained in a phosphorylated state until after the dissociation of insulin, and were dephosphorylated prior to their return to the plasma membrane. Thus the phosphorylated state of internalised receptors may also be regulated by insulin (Backer et al 1989).

The kinetics of insulin-receptor internalisation have

recently been described (Caro et al 1982).

Endocytosis of receptors appears to be a possible mechanism of reducing the number of insulin receptors at the cell surface through the lysosomal degradative pathway. Thus the potential of cell desensitisation to the action of insulin may be brought about by the down-regulation of the number of insulin receptors available. Receptor down-regulation may be caused by either an increased rate of receptor internalisation or an increased rate of removal of receptors from the plasma membrane. Although receptor down-regulation appears to be blocked by cycloheximide, receptor synthesis is not thought to be a contributory factor. It has, however, been suggested that protein synthesis may be involved in receptor recycling, blockage of which would result in receptor down-regulation. In addition, proteolysis of the insulin receptor at the plasma membrane may also be an important contributory factor in receptor degradation and down-regulation (Lipson et al 1988).

1.8.2 MECHANISM OF INSULIN ACTION

1.8.2.1 TYROSINE KINASE ACTIVATION

The 95kDa β -subunit of the insulin receptor is an insulin-regulated tyrosine kinase that is capable of autophosphorylation upon receptor activation (reviewed by Reddy and Kahn 1988, Kasuga et al 1982). Autophosphorylation of the β -subunit may then be responsible for activation of kinase activity for exogenous substrate phosphorylation.

Insulin stimulated the phosphorylation of tyrosine residues on a novel 60kDa G-protein (O'Brien et al 1989) in mouse NIH-3T3 cells.

Autophosphorylation occurs at several tyrosine residues in the β -subunit, although the regulatory tyrosine has not yet been identified (Czech 1985). Tyrosine 1150 has been proposed here. The insulin receptor is phosphorylated at 13 tyrosine residues in the β -subunit located in three clusters. Tyrosine kinase activation is an essential step in the action of insulin, and it has been reported that replacement of tyrosine for phenylalanine residues reduced both tyrosine kinase activity and glucose transport. The function of tyrosine phosphorylation appears to be the induction of a conformational change in the receptor that is then transmitted to the next step in insulin action (reviewed by Houslay 1985).

The tyrosine kinase domain begins 50 residues from the end of the insulin receptor transmembrane domain. Beginning at amino acid 991, this region has a Gly-x-Gly-x-x-Gly consensus sequence which is thought to be the ATP-binding site (Hunter and Cooper 1985). In addition, a lysine residue at position 1018 attracts the negatively charged third phosphate of ATP and directs it to tyrosine residues. An additional 90 residues distal to the carboxyl terminus of the tyrosine kinase domain are thought to regulate kinase activity (Hunter and Cooper 1985).

Insulin receptor tyrosine kinase activity appears to be regulated by tyrosine autophosphorylation in the β -subunit. Upon autophosphorylation of the insulin receptor kinase, the

presence of insulin is not required for its continued activity.

The role of tyrosine phosphorylation in insulin action is further supported by the apparent reduction in receptor autophosphorylation in STZ-diabetic rats, and in erythrocytes from insulin-resistant patients.

In addition to autophosphorylation of tyrosine residues (Kasuga et al 1982), the insulin receptor appears to phosphorylate other cellular proteins. In purified, solubilised receptor preparations, insulin predominantly phosphorylated at serine residues (White et al 1985). In contrast, insulin only phosphorylated at tyrosine residues in vitro, but in intact cells, serine and threonine residues were also phosphorylated. Serine phosphorylation was thought to decrease insulin action. It has been suggested that this phosphoserine-specific kinase is activated by the receptor tyrosine kinase, and may account for insulin-stimulated phosphorylation observed at serine residues elsewhere. This is further supported by the finding that the tyrosine kinase is activated before serine phosphorylation occurs (Czech 1985). The phosphorylated receptor is capable of being dephosphorylated by endogenous protein phosphatases. This is supported by the finding that vanadate, which inhibits tyrosine-specific phosphatases, increased receptor phosphorylation. Further, phosphotyrosyl residues are rapidly inactivated in intact cells, showing the presence of protein phosphatases (Sefton et al 1980). Seven forms of phosphotyrosyl-protein phosphatases have been purified from bovine brain (Jones et al 1989), and were distinct from the

type 1 and type 2 serine/threonine-protein phosphatases. Further, regulatory mechanisms for the control of these serine/threonine-protein phosphatases have been demonstrated. For instance, calcium ions activated pyruvate dehydrogenase phosphatase, and three heat-stable inhibitors of serine/threonine phosphatases are known to exist. Two inhibitor proteins of phosphotyrosyl-protein phosphatase have recently been identified and may be involved in their regulation (Ingebritsen 1989).

1.8.2.3 GUANINE NUCLEOTIDE REGULATORY PROTEINS

A number of guanine nucleotide regulatory proteins (G-proteins) have been isolated and characterised (Milligan 1988). For instance, G_s links the stimulation and G_i the inhibition of adenylate cyclase activity to the hormone receptors. G-proteins consist of three distinct subunits α , β and γ . The α -subunit comprises the GTP-binding site, sites that interact with the receptor and target systems and a Mg^{2+} -binding site. The α -subunits of G_i and G_s can be identified separately using cholera and pertussis toxins, which cause the NAD^+ -dependent ADP-ribosylation of G_s and G_i respectively (Houslay 1985).

Activation of G-proteins by hormone-receptor complexes results in the release of the activated α -subunit of the G-protein. In the case of the G_s , the activated α -subunit interacts with and activates the catalytic unit of adenylate cyclase. G_i is thought to inhibit adenylate cyclase activity by two mechanisms. Firstly, by releasing an activated

α -subunit which binds to the catalytic subunit of adenylate cyclase, and secondly by releasing its β - γ complex. This complex, which is structurally very similar to that in G_s , inhibits G_s dissociation, thereby inhibiting its stimulatory action on adenylate cyclase.

The α -subunits of all G-proteins also exhibit a GTPase activity, which is thought to be responsible for the deactivation of the dissociated and activated α -subunit. More recently, phosphorylation of α - G_i has been demonstrated, concomitant with the functional inactivation of G_i to inhibit adenylate cyclase activity (Pyne et al 1989).

Insulin has been shown to stimulate a GTPase activity in human platelets, and appears to be distinct from other G-proteins. Insulin is now thought to inhibit adenylate cyclase activity not via G_i , but via G_{ins} , a novel G-protein thought to mediate the effects of insulin on many systems (Houslay 1986). The α -subunit of G_{ins} has been identified as a 25kDa molecule.

Other factors aside from G_{ins} are also involved in mediating the ability of insulin to inhibit adenylate cyclase and activate cyclic AMP phosphodiesterases. Activation of the plasma membrane cyclic AMP phosphodiesterase appears to be as a result of phosphorylation of intrinsic serine residues. Although the mechanism of this activation is not yet known, G_{ins} has been implicated (Heyworth and Houslay 1983, Heyworth et al 1983a & 1983b).

1.8.2.4 SECOND MESSENGERS OF INSULIN ACTION

The ability of insulin to antagonise the actions of hormones such as glucagon, that act via cyclic AMP mechanisms, led to the belief that insulin may be mediating its effects by lowering cyclic AMP levels. The possibility that many of the effects of insulin are as a result of changes in intracellular cyclic AMP levels is a much debated area. Although the mechanism by which insulin may lower cyclic AMP exists, that is, inhibition of adenylate cyclase and stimulation of cyclic AMP phosphodiesterase, several studies have reported controversial results.

Kuo and De Renzo (1969) demonstrated an insulin-stimulated reduction in cyclic AMP levels in adipocytes in the presence of lipolytic agents (Fain et al 1971). Insulin was shown to activate membrane-bound high affinity cyclic AMP phosphodiesterase, but not cytosolic phosphodiesterase in vivo, although the same effects were not observed in vitro (Thompson et al 1973), and may therefore explain the reduction in cyclic AMP levels seen above. A similar reduction in cyclic AMP levels in response to insulin was noted in hepatocytes, although this was attributed to the alteration in adenylate cyclase activity (Iliano and Cuatrecasas 1972). The role of the G-proteins in insulin-stimulated activation of cyclic AMP phosphodiesterase and inhibition of adenylate cyclase is well documented (Houslay 1985). In contrast to these reports, Tarui et al (1976) reported that the effects of insulin on diaphragm muscle were not mediated by alterations

in either cyclic AMP or GMP levels.

More recently, protein kinase C activation has been implicated in the stimulation of lipogenesis by insulin in isolated rat adipocytes since the effect was abolished in the presence of the protein kinase inhibitor acridine orange (Smal et al 1989). Similarly, protein kinase C was activated in response to insulin in BC3H-1 myocytes (Acevedo-Duncan et al 1989). Further, insulin and phorbol esters phosphorylated several proteins that are markers of protein kinase C activation, suggesting that insulin acted via protein kinase C activation (Vila et al 1989). By the use of specific inhibitors, it was shown that diacylglycerol production was involved in the insulin-stimulated increase in protein synthesis in 3T3 fibroblasts (Hesketh et al 1988). Further, glucose transport, stimulated by insulin was dependent on protein kinase C activation (Cooper et al 1989).

In contrast to these reports, however, Andersen et al (1988) have reported that PMA and insulin-stimulated processes acted in an additive manner, and were therefore proceeding by distinct mechanisms. Taylor et al (1985) showed that insulin did not influence phosphoinositide formation in isolated hepatocytes. However, insulin stimulated the de novo synthesis of phosphatidylinositol and phosphatidylinositol-4,5-bisphosphate, but had no effect on phosphoinositide breakdown in adipocytes (Pennington and Martin 1985), showing that protein kinase activation was not involved in insulin action. Farese et al (1988a & 1988b) have also demonstrated an increase in PIP_2 , phosphatidic

acid and diacylglycerol synthesis, an effect that was abolished by protein synthesis inhibitors. Thus insulin stimulated the biosynthesis of phospholipids, but not their hydrolysis, which is contrary to the findings of other reports.

1.8.3 INSULIN MEDIATOR HYPOTHESIS

The rapid responses observed upon insulin binding to its receptor are thought to be mediated by a second messenger or chemical mediator.

A chemical mediator of insulin action was first proposed by Larner et al (1979) who showed that a substance extracted from insulin-treated skeletal muscle inhibited cyclic AMP-dependent protein kinase. A similar extract stimulated pyruvate dehydrogenase (PDH) activity in adipocyte mitochondria (Jarett and Seals 1979). In addition, treatment of a mixture of plasma membranes and mitochondria with insulin also stimulated PDH activity (Seals and Jarett 1980). This low molecular weight mediator (1000-2000kDa) was also generated upon treatment of liver plasma membranes with insulin, and stimulated both adipocyte and liver mitochondrial PDH activity (Saltiel et al 1981). Further, this effect was dependent on the concentration of insulin used to generate the mediator substance.

The insulin mediator substance has subsequently been isolated and purified from rat skeletal muscle (reviewed by Cheng et al 1984), plasma membranes (reviewed by Seals and Czech 1982), hepatocytes (reviewed by Saltiel 1987),

adipocytes (Kiechle et al 1980), IM-9 lymphocytes (Jarett et al 1980), hepatoma cells (Parker et al 1982) and from human mononuclear cells and placental plasma membranes (Suzuki et al 1984a). In its purified form, all the putative insulin mediators isolated as yet appear to be low molecular weight (1000-2000kDa) carbohydrate compounds that are acid-and heat-stable (Saltiel 1987, Suzuki et al 1984b), but are reported to be susceptible to hydrolytic cleavage (Suzuki et al 1984b, Zhang et al 1983).

Reports of a biphasic effect (inhibitory and stimulatory) with different concentrations of the insulin mediator extract on numerous systems have led to the idea of the existence of a family of insulin mediators (Larner et al 1979, Suzuki et al 1987). Dose-response experiments with the insulin mediator extract have supported this claim, and it was suggested that the stimulating effects of the mediator were more active at lower concentrations than was the inhibitory activity. The generation of multiple insulin mediators has further been supported by subsequent purification of the extract. Inhibitory and stimulatory activities have now been separated electrophoretically (Cheng et al 1985) and by column chromatography (Larner et al 1988). Further, multiple stimulatory activities with the same biological actions have also been detected (Romero et al 1988). The generation of different insulin mediators may be attributed to tissue differences.

Purification of the insulin mediator extracts have led to the partial characterisation of the structure of the inositol phosphate glycan (IPG). Although some differences

have been reported (Larner et al 1988), the IPG consists of an inositol monophosphate linked to glucosamine, four residues of galactose and two additional phosphate groups that are not linked to inositol (Mato et al 1987b, Saltiel 1987). In addition, three different phosphorylated forms of the IPG have recently been purified, and it was shown that the biological activity of each of these was dependent on their phosphorylation state (Merida et al 1988).

The mechanism by which insulin elicits IPG generation is not fully clear as yet. Early reports led to the belief that a proteolytic reaction was involved (Larner et al 1982, Cherqui et al 1982), and although this theory has not been entirely disproved, it is not thought to be valid.

A mechanism by which treatment of various tissues with insulin generated the insulin mediator has recently been proposed by Saltiel and co-workers. Two chemically related carbohydrate substances and diacylglycerol were purified from an extract prepared by treatment of plasma membrane fractions with insulin (Saltiel and Cuatrecasas 1986, Saltiel et al 1986). Further, this insulin mediator could also be generated from plasma membranes incubated with a phosphatidylinositol-specific phospholipase C (PI-specific PL_C) from *Staphylococcus aureus* (*S. aureus*), which selectively hydrolyses glycosyl-phosphatidylinositol to release compounds that are covalently linked to the phosphatidylinositol moiety by a glycan anchor (Low and Saltiel 1988). This led to the suggestion that insulin activated an endogenous PI-specific PL_C which, upon hydrolysis of the phosphatidylinositol-glycan (PI-glycan),

resulted in the formation of the inositol phosphate-glycan (IPG) mediator of insulin action (Saltiel et al 1986). Myristate diacylglycerol (DAG) was also produced during the course of this reaction (Saltiel et al 1987), and was not as a result of de novo DAG synthesis, as was proposed by Farese et al (1988a). In addition, increased synthesis of phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol glycan by insulin may represent replenishing of the precursor stores that are hydrolysed by insulin (Farese et al 1988b), and further supports the role of the IPG in insulin action.

The finding that EGF and IGF-I also stimulate the release of IPG implies that tyrosine kinase activation may be involved in PI-glycan hydrolysis (Farese et al 1988c). The observation that IPG generation by liver plasma membranes is markedly stimulated in the presence of ATP and Mn^{2+} , similar to the requirements for tyrosine kinase phosphorylation, supports the claim that tyrosine kinase rather than serine or threonine phosphorylation is involved (Suzuki et al 1987). Since insulin failed to stimulate IPG generation from diabetic livers (Amatruda and Chang 1983), its release appears to be a specific effect of insulin rather than of tyrosine kinase activation alone.

The precursor PI-glycan has been isolated and characterised by several groups (Mato et al 1987a), and appears to be almost exclusively located on the external surface of the rat liver plasma membrane (Alvarez et al 1988). In addition, treatment of this purified glycopospholipid with either insulin or PI-specific PL_C

from liver plasma membranes (Fox et al 1987), *S. aureus* (Kelly et al 1986) or *Bacillus cereus* (Merida et al 1988) resulted in the release of its polar head group, the insulin mediator. A striking resemblance became apparent at this point between insulin mediator generation and other systems such as alkaline phosphatase and heparan sulphate, which are also anchored to the plasma membrane by glycan structures (Low and Saltiel 1988). In addition, a novel glycan PI-specific PL_C that catalyses the conversion of membrane form variant surface glycoproteins (mfVSG) has been isolated from *Trypanosoma brucei* (Fox et al 1986). The glycolipid moiety of mfVSG contains dimyristyl-phosphatidylinositol glycosidically linked to glucosamine, and it is coupled to the polypeptide by additional carbohydrate, phosphate and ethanolamine, and forms an amide linkage with the carboxy terminus amino acid residue (Ferguson et al 1985a and b). The PL_C has been reported to cleave a phosphodiester bond in this structure. A phospholipid labelled with 3H -inositol, 3H -glucosamine and 3H -myristate was recently reported to be hydrolysed by insulin-stimulated PL_C (Farese et al 1988c), which is consistent with the components of the IPG.

The PI-specific PL_C has also been purified. Fox et al (1986) purified the 40K PL_C from *T. brucei*. The enzyme showed a specificity for molecules containing carbohydrate covalently linked to PI-glycans. The enzyme did not require any specific ions for its activity, and was therefore calcium ion independent, and was stimulated by thiol-reducing agents and inhibited by thiol-chelating compounds. A 62kDa PI-specific PL_C has subsequently been

characterised from liver plasma membranes (Fox et al 1987) which appears to be associated with membrane lipid, and showed identical properties to the PL_C isolated from *T. brucei* (Fox et al 1986).

Numerous reports have accumulated showing the homology between the actions of insulin and its mediator on various systems. The effects of insulin that are mimicked by the insulin mediator are summarised in Table 4.

TABLE 4 : "INSULIN-LIKE" EFFECTS OF THE INSULIN MEDIATOR
(adapted from Gottschalk and Jarett 1985, Cheng and Larner 1985)

<u>SOURCE and METABOLIC SYSTEM</u>	<u>EFFECT</u>	
	<u>MEDIATOR</u>	<u>INSULIN</u>
A) CELL-FREE SYSTEMS		
<u>CYTOPLASM</u>		
Cyclic AMP-dependent protein kinase	-	-
Glycogen synthase	+	+
Acetyl CoA carboxylase	+	+
<u>CELL MEMBRANE</u>		
Adenylate cyclase (hormone-stimulated)	-	-
Ca ²⁺ , Mg ²⁺ -ATPase	-	+
Na ⁺ , K ⁺ -ATPase	+	+
<u>ENDOPLASMIC RETICULUM</u>		
Cyclic AMP phosphodiesterase	+	+
Glucose-6-phosphatase	-	-
<u>MITOCHONDRIA</u>		
Pyruvate dehydrogenase	+	+
Ca ²⁺ transport	+	+
<u>NUCLEUS</u>		
Transcription of β -casein gene	+	
RNA synthesis	+	+
B) INTACT CELL SYSTEM		
<u>RAT ADIPOCYTE</u>		
Hormone-stimulated cAMP concentration	-	-
Lipogenesis	+	+
Antilipolysis	+	+
Low K _M cAMP phosphodiesterase	+	+
<u>RAT HEPATOCYTE</u>		
Lipogenesis	+	+
Down-regulation of insulin receptor	+	+
Adenylate cyclase	-	-
<u>HUMAN MONOCYTES</u>		
Glucose-6-phosphatase	-	-
<u>IM-9 LYMPHOCYTES</u>		
Adenylate cyclase	-	-
<u>RAT LIVER</u>		
Phospholipid methyltransferase	-	-
Steroid metabolism	+	?

The IPG in general, appears to act by activation of protein kinases and phosphatases to modulate the phosphorylation state, and therefore activity of its target enzymes. Such a phosphorylation-dephosphorylation control mechanism has been proposed (Larner et al 1979).

Both cyclic AMP-dependent and independent effects of insulin were mimicked by the IPG (Alemany et al 1987), indicating that mediator generation occurred early in insulin action, prior to second messenger activation.

The effects of the insulin mediator on pyruvate dehydrogenase (PDH) activation have been documented extensively (Jarett and Seals 1979, Saltiel et al 1981). PDH is a multi-enzyme complex comprising the five enzymes pyruvate decarboxylase, dihydrolipoyl acetyltransferase, dihydrolipoyl dehydrogenase, a cAMP-independent protein kinase and a phosphoprotein phosphatase. The activity of the complex is regulated by the extent of phosphorylation of the α -subunit of the decarboxylase, which is in turn determined by the competing effects of the PDH kinase and PDH phosphatase. The dephosphorylated enzyme is the active form. Thus the mechanism by which insulin and insulin mediator stimulate PDH activity could be either by inhibition of the kinase or activation of the phosphatase. PDH activation was blocked in the presence of sodium fluoride, an inhibitor of PDH phosphatase, thus showing that both insulin and the insulin mediator stimulated PDH activity by activation of the PDH phosphatase (Suzuki et al 1984a), and therefore caused a dephosphorylation of the α -subunit. In addition, inhibition of the PDH kinase had no effects on the ability

of insulin and insulin mediator to activate PDH, showing that the kinase was not involved (Macaulay and Jarett 1985). An ethanol-insoluble PDH inhibitor has also been identified in the insulin mediator extract, and is thought to be prevalent at higher concentrations of insulin mediator (Suzuki et al 1987, Saltiel et al 1982).

Both insulin and the insulin mediator are known to exert effects on the cyclic AMP system. Adenylate cyclase activity was inhibited by the insulin mediator (Pyne and Houslay 1988, Saltiel 1987, Malchoff et al 1987, Macaulay and Jarett 1985) by modulating its catalytic subunit (Malchoff et al 1987). The insulin mediator also inhibited the catalytic subunit of cAMP-dependent protein kinase, and at a site distinct from the ATP-binding sites (Villalba et al 1988, Malchoff et al 1987). Low K_m , but not high K_m , cyclic AMP phosphodiesterase activity was increased by the insulin mediator (Kiechle and Jarett 1981, Parker et al 1982), but neither peripheral plasma membrane or dense-vesicle cyclic AMP phosphodiesterase were affected (Pyne and Houslay 1988).

Thus the mechanism exists for modulation of intracellular cyclic AMP levels by insulin. The insulin mediator has also been reported to lower glucagon-elevated, although not basal, cyclic AMP levels (Zhang et al 1983, Alvarez et al 1987).

The insulin mediator was reported to inhibit the stimulatory effects of isoprenaline on phospholipid methyltransferase activity in adipocytes (Kelly et al 1986, 1988) by inhibiting the isoprenaline-induced phosphorylation

and therefore activation of phospholipid methyltransferase (Kelly et al 1987). In contrast to this, the same extract failed to mimic the stimulatory effects of insulin on glucose transport (Kelly et al 1987). Thus a dissociation of the effects of insulin and its mediator were observed (Jarett et al 1985). This discrepancy was resolved by Standaert et al (1988), who showed that diacylglycerol, its structural analogues and insulin all stimulated glucose transport in BC3H-1 myocytes, and therefore implied a role of protein kinase C activation in insulin-stimulated glucose transport.

1.8.4 PROPOSED MECHANISM OF INSULIN ACTION

Based on the various steps that follow the binding of insulin to its receptor, a possible pathway for insulin action has been proposed by Brautigan and Kuplic (1988) (Figure 4) :

Insulin binds to the α -subunit of its receptor, causing a conformational change, and activates an intrinsic tyrosine kinase in the β -subunit. This activated receptor kinase then phosphorylates a tyrosine residue in a regulatory G-protein, possibly G_{ins} . The α -subunit of this G-protein then activates a specific phospholipase C, which in turn catalyses the phosphodiesteratic hydrolysis of a membrane-bound phosphatidylinositol-glycan to release myristate diacylglycerol and a carbohydrate inositol phosphate-glycan moiety. This IP-glycan is the proposed mediator of insulin action that presumably controls target

Abbreviations : cAMP=cyclic AMP, cAMP PDE=cyclic AMP-dependent phosphodiesterase, DAG=diacylglycerol, Gins=guanine nucleotide regulatory protein specific for insulin, IPG=inositol phosphate glycan, PI-PLC=phosphatidylinositol-specific phospholipase C, Tyr-P=insulin receptor tyrosine kinase.

The diagram illustrates the insulin signaling pathway. Insulin binds to its receptor, a dimer of α and β subunits. This activates the receptor's tyrosine kinase activity, leading to autophosphorylation of the β subunits (Tyr-P). The activated receptor then phosphorylates insulin receptor substrate (IRS), which is also phosphorylated (Tyr-P). IRS activates phosphatidylinositol 3-kinase (PI 3-K), which produces phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 recruits and activates phosphoinositide-dependent kinase-1 (PDK1). PDK1 then phosphorylates various substrates, including protein kinase B (PKB) and serine/threonine kinases, leading to their activation. Activated PKB inhibits glycogen synthase kinase-3 (GSK-3), which in turn activates glycogen synthase (GS). Activated serine/threonine kinases also activate GS. The diagram shows the flow from insulin binding to the final activation of glycogen synthase (GS) and the inhibition of glycogenolysis (GLYCOGEN PHOSPHORYLASE).

enzymes by a phosphorylation-dephosphorylation mechanism. The role of serine/threonine phosphorylation by insulin in this pathway has not yet been recognised, although it has been suggested that the insulin mediator substance itself may be involved in the phosphorylation of serine/threonine kinases rather than the intrinsic tyrosine kinase (Czech et al 1988).

AIMS OF THE PRESENT PROJECT :

- 1) To observe the effects of GH administration to isolated hepatocytes on steroid metabolism and to relate this to results reported in the intact animal.
 - 2) To attempt to elucidate the mechanism of action of GH on steroid metabolism.
 - 3) To determine the effects of insulin on steroid metabolism and its interaction with GH.
 - 4) To determine the effects of dexamethasone on steroid metabolism and its interaction with GH.
 - 5) To determine the effects of thyroxine on steroid metabolism and its interaction with GH.
 - 6) To determine the effects of GH on steroid metabolism in the presence of dexamethasone AND thyroxine.
 - 7) To determine the effects of the insulin mediator on hepatic steroid metabolism and to correlate this with the results obtained with insulin.
 - 8) To purify the insulin mediator extract and identify the constituents of the insulin mediator.
- The primary aim of this project was therefore to

elucidate the effects of GH on hepatic steroid metabolism, and to determine the culture conditions that would manifest the feminising effect expected of GH from in vivo studies. The second part of this study comprised of studies with the putative insulin mediator isolated from hepatocytes in an attempt to determine if this mediator was involved in insulin action on hepatic steroid metabolism.

MATERIALS AND METHODS

MATERIALS AND METHODS

2.0 EXPERIMENTAL ANIMALS

Mature male Wistar rats bred in the department were used throughout this study. The animals were housed under light-and temperature-controlled conditions (lights on 0800-2000; $19\pm 1^{\circ}\text{C}$) and were allowed access to food (CRM Nuts, Labsure, Croydon) and water ad libitum. The rats were 10-12 weeks old at the time of experimentation, and weighed between 350-400g.

2.1 PREPARATION OF RAT HEPATOCYTES

Hepatocytes were isolated using a modification of the two-step collagenase perfusion technique described by Seglen (1973). The animal was anaesthetised under 3.5% halothane : 400ml/min oxygen : 800ml/min nitrous oxide using a MiniBoyle anaesthetic machine. The abdomen was then cut open and a cannula inserted into the hepatic portal vein, ensuring that it was placed before any branch points in the vein so as to allow adequate perfusion of the whole liver. The liver was perfused using a Watson-Marlow 501U perfusion pump with calcium-free Hank's balanced salt solution (HBSS) at a rate of 90ml/min until the liver reached twice its original size, at which point the vena cava was punctured. The liver was perfused for a further 7-8 minutes at a rate of 60ml/min. After 10 minutes, the perfusion was switched over to calcium-collagenase buffer (calcium-free HBSS supplemented

with 4mM calcium chloride and 0.5mg/ml collagenase). The calcium-collagenase buffer was recirculated for about 12.5 minutes, until the liver appeared to be sufficiently digested, at which point the liver was removed from the animal. The cells were then gently "combed-out" in HBSS and the resulting cell suspension filtered through two layers of gauze to remove connective tissue and undigested liver, and centrifuged at 200g for 2min in a refrigerated DAMON/IEC DPR 600 centrifuge to remove cell debris from liver parenchymal cells. The isolated hepatocytes were then resuspended in incubation medium (calcium-free HBSS, 1g/l glucose, 100mg/ml MgSO_4 , 100mg/ml MgCl_2 and 185mg/ml CaCl_2), and counted using a haemocytometer. Yield of cells was about 10^8 cells/g liver, and cell viability exceeded 90% in all cases, as assessed by the trypan blue exclusion method.

2.2 PREPARATION OF RAT LIVER MICROSOMES

The method used is a modification of that described by Berg and Gustafsson (1973). The animal was killed by CO_2 asphyxiation, and the liver removed, rinsed in ice-cold 0.25M sucrose, blotted dry and weighed. All buffers were kept at 4°C throughout. The liver was finely chopped, and portions of liver were homogenised in four volumes of sucrose buffer using a Potter-Elvehjem homogeniser with a loose-fitting Teflon pestle at 10,000 rpm. The homogenate was then centrifuged in a refrigerated SORVALL RC-5B centrifuge for 15 minutes at 12,500g to sediment mitochondria and nuclei. This leaves the postmitochondrial

supernatant fraction. Calcium chloride is then added to this postmitochondrial supernatant at a final concentration of 8mM, mixed thoroughly, and left to stand at 4°C for 5 minutes. The microsomes are then pelleted by centrifugation at 27,000g for 20 minutes. The supernatant is then decanted, the pellet resuspended in 0.25M sucrose, and stored on ice until use. Tissue protein content was determined as described in Section 2.4.

2.3 PREPARATION OF RAT LIVER MITOPLASTS

The method used for the preparation of rat liver mitoplasts was as described by Saltiel (1984). The animals were killed by CO₂ asphyxiation and the liver was washed in 20mM potassium phosphate buffer pH 7.4, homogenised in 10 volumes of buffer A (0.075M sucrose, 1mM Tris, 0.1mM EDTA, pH 7.2) and centrifuged at 500g for 10 minutes. The supernatant was then re-centrifuged at 500g for a further 10 minutes. This pellet was homogenised in an equal volume of buffer A, and the two centrifugation steps repeated. The pellet from this step consisted of whole mitochondria. The outer mitochondrial membranes were then detached by swelling in 10 volumes of 20mM potassium phosphate pH 7.2 supplemented with 0.02% bovine serum albumin (Buffer B), gradually added over 45 minutes at 4°C with gentle shaking every 5 minutes. This suspension was centrifuged at 40,000g for 20 minutes at 4°C in order to shear the outer membranes, and the resultant pellet resuspended in buffer B. This mixture of inner and outer mitochondrial membranes was then

separated by further centrifugation at 1900g for 15 minutes. The resulting pellet, consisting of inner mitochondrial ghosts (mitoplasts) highly enriched in pyruvate dehydrogenase activity, was resuspended in 20mM potassium phosphate buffer pH 7.0, and stored at -20°C for up to two weeks with negligible loss of enzyme activity. Tissue protein content was determined as per Section 2.4.

2.4 DETERMINATION OF TISSUE PROTEIN CONTENT

Tissue protein content was determined by the method of Lowry et al (1951), and follows the formation of a coloured complex between the alkaline copperphenol reagent and the tyrosine and tryptophan residues of the protein measured spectrophotometrically at 750nm.

To 1ml sample of protein solution (containing approximately 150µg protein) is added 2ml of freshly prepared reagent (50 parts 4% w/v Na_2CO_3 in 0.1N NaOH : 1 part 1% w/v potassium disodium tartarate : 1 part 0.5% w/v hydrated copper sulphate) and left to stand for 10 minutes at room temperature. 0.3ml of 1N Folin Ciocalteu reagent is then added, rapidly mixed, and left to stand for 30 minutes. Optical density was then determined at 750nm, and the protein content of the unknown samples calculated from a standard curve prepared using 0.2ml to 1.0ml aliquots of 200µg/ml bovine serum albumin.

2.5 PRETREATMENT OF HEPATOCYTES

2.5.1 PREINCUBATION OF HEPATOCYTES OVER 0-60 MINUTES

Hepatocytes (2×10^7 cells/incubation) were incubated in glass tubes in hormone-free Ham's F-10 culture medium supplemented with 0.1% bovine serum albumin in a final volume of 10ml at 37°C in a rotary shaking waterbath. Hormones were subsequently added in the minimum volume possible, control incubations receiving an equivalent volume of drug vehicle alone. Hormonal additions were as follows :

- a) 10^{-9}M - 10^{-7}M growth hormone in distilled water
- b) 10^{-11}M - 10^{-6}M insulin in 0.1N HCl
- c) 10^{-8}M dexamethasone in acetone
- d) 10^{-8}M - 10^{-6}M thyroxine in 0.01N NaOH
- e) 10^{-10}M - 10^{-8}M insulin-like growth factor I in H_2O

None of these additions altered the pH of the incubation system significantly.

2.5.2 PREINCUBATION OF HEPATOCYTES OVER 24, 48 AND 72HR

Hepatocytes were plated (NUNCCLON 9cm diameter petri dishes) at a cell density of 2×10^7 cells/incubation in 10ml Ham's F-10 culture medium supplemented with 100µg penicillin / streptomycin and 2% Ultrosor G (LKB), which is a serum substitute to promote cell attachment and monolayer formation, and incubated at 37°C under 95% air and 5% CO_2 in a humidified incubator (Forma Scientific Model 3028). After 24h of incubation, the medium was replaced with Ham's F-10

culture medium supplemented with 0.1% bovine serum albumin and 100µg penicillin/streptomycin. Hormones were added to the plated cells (Section 2.5.1) after 24h of incubation, and incubated for a further 24, 48 and 72 hours, as described by Hussin and Skett (1986). In some cases, pretreated cells are then further incubated with growth hormone.

2.6 ASSAY OF STEROID METABOLISM

2.6.1 ASSAY OF STEROID METABOLISM IN HEPATOCYTES

After pretreatment with hormone, cell suspensions were centrifuged at 200g for 2min (4°C) and the supernatant was discarded. Hepatocytes were then washed and resuspended in incubation medium, and counted prior to assaying for steroid-metabolising activity.

The assay used is a modification of that described by Gustafsson and Stenberg (1974). Hepatocytes (6×10^6) were incubated with 500µg 0.1µCi $[4-^{14}\text{C}]$ -androst-4-ene-3,17-dione in a final volume of 3ml in incubation medium. Incubations were carried out in a rotary shaking waterbath at 37°C for 30min in triplicate, after which the reaction was stopped by the addition of 5ml Folch solution (chloroform : methanol, 2:1 v/v) and 1ml 0.9% NaCl to aid extraction of metabolites into the organic layer. The tubes were gently shaken and the two phases allowed to separate overnight. The tubes were then centrifuged at 500g for 5min in order to achieve complete separation of the two phases. The lower organic

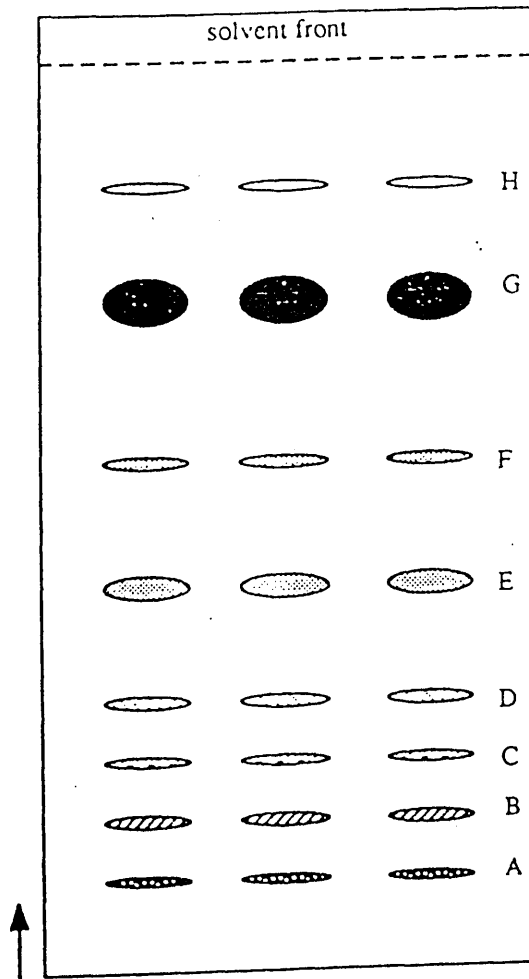
layer was then removed and dried under a stream of nitrogen at 40°C. The extract was redissolved in a few drops of chloroform and sonicated for about 30 seconds in an ultrasonic waterbath. Samples were then spotted onto 0.25mm Merck silica gel thin layer chromatography plates (20cm x 10cm), and separation of metabolites was achieved by running the plates in a mobile phase consisting of chloroform : ethyl acetate (4:1 v/v) in tanks equilibrated with the solvent for at least one hour. The plates were then dried, and metabolites were located by autoradiography (Figure 5). The identity of the metabolites has previously been characterised by gas chromatography -mass spectrometry (Gustafsson and Stenberg 1974). Metabolites were then scraped into counting vials, suspended in 3ml of Ecoscint liquid scintillant and counted in a Packard Tri-Carb 2000CA Liquid Scintillation Analyser in dpm mode using a commercial quenched standard set.

2.6.2 ASSAY OF STEROID METABOLISM IN LIVER MICROSOMES

The assay was essentially as that described in section 2.6.1 except that cofactors were also included in the reaction system. Microsomes were incubated in 0.25M Sucrose - 0.1M Tris buffer (pH 7.4) with 500µg, 0.1µCi [4-¹⁴C]-androst-4-ene-3,17-dione and 110µl cofactor mixture (3mg NADP, 3.9mg sodium isocitrate, 6µg manganese chloride and 10µl isocitrate dehydrogenase added immediately before starting the incubation) in a final volume of 3ml. The incubation was carried out at 37°C for 30min, and metabolite

FIGURE 5 : THIN LAYER CHROMATOGRAPHY OF ANDROSTENEDIONE METABOLITES.

- A. Start
- B. Androst-4-ene-7 α -ol-3,17-dione
- C. Androst-4-ene-6 β -ol-3,17-dione
- D. Androst-4-ene-16 α -ol-3,17-dione
- E. Testosterone/Epitestosterone
- F. 5 α -androstane-3 α (β)-ol-17-dione
- G. Unchanged substrate (Androst-4-ene-3,17-dione)
- H. 5 α -androstane-3,17-dione



formation was determined as detailed above.

2.6.3 EVALUATION OF ENZYME ACTIVITY

Activities of each of the enzymes was determined by the amount of metabolite formed, and then calculated using the following formula :

$$\begin{aligned} &\text{pmoles metabolite / minute / million cells (mg protein)} \\ &= \quad S \times \frac{\text{cpm}_M}{\text{cpm}_T} \times \frac{1}{t} \times \frac{1}{n} \times \frac{1}{\text{MW}} \end{aligned}$$

where S = μg substrate (500 μg)

cpm_M = cpm metabolite

cpm_T = cpm total

t = incubation time (30min)

MW = molecular weight of substrate (280)

n = number of cells (millions) OR mg protein
(microsomes)

Thus enzyme activity is expressed as pmoles/min/million cells (hepatocytes) or as pmoles/min/mg protein (microsomes). The actual calculation was performed using a custom-made computer program.

2.7 MEASUREMENT OF INTRACELLULAR CYCLIC AMP

The intracellular cyclic AMP concentration was determined by the radioimmunoassay described by Brooker et al (1979). Following incubation with growth hormone over various time periods, cell samples were pooled, counted and then centrifuged at 200g for 2min at 4°C. The supernatant

was removed and the cells were resuspended in sodium acetate buffer to give an approximate concentration of 10^7 cells/ml (approximately 20nM cAMP). The cells were then placed in a boiling waterbath for 10-15 minutes to denature the protein and extract the cAMP into solution, and then centrifuged at 4000g for 10min. The supernatant was stored at -20°C prior to assaying for cyclic AMP content. Cyclic AMP standards (10^{-10} to 10^{-6} nM) were also prepared in triplicate in sodium acetate buffer.

Cyclic AMP content was determined in 100 μ l aliquots of both standards and unknown samples. To each 100 μ l aliquot is added :

- 1) 10 μ l of a freshly prepared mixture of triethylamine and acetic anhydride (2:1 v/v) to acetylate the samples thereby increasing sensitivity, followed rapidly by vortex mixing;

- 2) 150 μ l of a 10^{-4} dilution of anti-cyclic AMP antiserum (in 0.1% BSA) which was raised in goats treated with human serum albumin conjugated with succinyl cyclic AMP;

The specificity of the antiserum was tested by measuring the ability of a range of adenine and guanine nucleotides to compete with $[^{125}\text{I}]$ -cyclic AMP for binding to the antiserum. The antiserum was relatively specific for cyclic AMP, with 10^3 fold (cGMP) and 10^4 fold (AMP, ADP, ATP) higher concentrations of the other nucleotides required to produce 50% displacement of the $[^{125}\text{I}]$ -cyclic AMP.

- 3) 100 μ l of a 1/7000 dilution of $[^{125}\text{I}]$ -cyclic AMP (3000cpm per sample).

Samples were then mixed and incubated for 16h at 4°C. After this incubation, 0.5ml of a suspension of activated charcoal (1% w/v) in 100mM potassium phosphate buffer pH 6.4 containing 0.25% BSA was added to each of the samples in order to remove unbound nucleotide. The samples were then mixed and the charcoal sedimented by centrifugation at 4000g for 10min at 4°C. 600µl of this supernatant was taken to assess the antibody-bound $[^{125}\text{I}]$ -cyclic AMP, and counted in a gamma counter (LKB 1275 Gamma counter).

Standard calibration curves were plotted as radioactivity bound (cpm) against concentration of cyclic AMP or as B/B_0 against concentration of cyclic AMP, where B and B_0 represent the amount of $[^{125}\text{I}]$ -cyclic AMP bound in the presence or absence of unlabelled cyclic AMP respectively. In the absence of cyclic AMP, approximately 30% of the total added $[^{125}\text{I}]$ -cyclic AMP was bound. Amounts of cyclic AMP were then determined in the unknown samples by using the standard curve and expressed as pmoles of cyclic AMP per million cells.

2.8 PREPARATION OF THE INSULIN MEDIATOR EXTRACT

2.8.1 PREPARATION OF INSULIN MEDIATOR FROM RAT LIVER MICROSOMES

The insulin mediator extract was prepared using the method of Suzuki et al (1987). Rat liver microsomes (2.5mg protein) were incubated with and without insulin in 50mM Tris-HCl buffer pH 7.4 supplemented with 0.01% BSA, 1mM ATP,

3mM MnCl_2 , 1M CaCl_2 at 37°C for 5min in a total volume of 2ml. 2ml of 1M formic acid containing 5mM EDTA was then added to the reaction mixture and the tubes placed in a boiling waterbath for 5min. After centrifugation (15000g, 10min, 4°C) the denatured precipitate was removed and the supernatant treated with 0.2mg/ml activated charcoal for 10min at 4°C. The supernatant was then separated from the charcoal by centrifugation (15000g, 10min, 4°C) and lyophilised at -50°C overnight. The resulting white powder was resuspended in 1ml of 1mM formic acid pH 3.5 and labelled as the original insulin mediator extract.

These original extracts were then twice extracted with 2ml of chloroform : methanol (2:1 v/v) at 4°C for 10min. The soluble fraction was removed and the insoluble residue extracted with 2ml of ethanol at 4°C for 10min. The soluble fraction was discarded and the insoluble precipitate was dried under a stream of nitrogen. This precipitate was redissolved in 1ml of 1mM formic acid pH 3.5, and the insoluble residue removed by centrifugation. The resulting supernatant, consisting of the partially purified insulin mediator extract, was then applied to a 1x2cm AG1x8 ion-exchange resin equilibrated with 1mM formic acid pH 3.5. The column was then eluted sequentially with 6ml each of 1mM formic acid; 0.025M, 0.1M, 0.2M, 0.5M, 1.0M ammonium formate and 1.0M formic acid. The eluates were collected, lyophilised at -50°C and redissolved in 1mM formic acid pH 3.5 immediately before assay.

2.8.2 PREPARATION OF INSULIN MEDIATOR FROM RAT HEPATOCYTES

The insulin mediator extract was prepared from rat hepatocytes essentially as described above in section 2.8.1, except that hepatocytes (1.2×10^7 cells) were incubated with or without insulin in a final volume of 2ml of incubation medium supplemented with 0.1% BSA.

2.9 PRETREATMENT WITH THE INSULIN MEDIATOR EXTRACT

2.9.1 PRETREATMENT OF HEPATOCYTES WITH INSULIN MEDIATOR

Hepatocytes (2×10^7 cells) were incubated at 37°C with varying doses of the insulin mediator extract in incubation medium supplemented with 0.1% BSA in a final volume of 5ml. After incubating for the required time (0 to 60min), the cell suspension was centrifuged (200g, 2min, 4°C) and cells resuspended in incubation medium prior to being assayed for steroid metabolism (Section 2.6.1).

2.9.2 PRETREATMENT OF LIVER MICROSOMES WITH INSULIN MEDIATOR

Rat liver microsomes were incubated with varying concentrations of the insulin mediator extract for 30min and at 37°C in a final volume of 5ml in 0.25M Sucrose-0.25M Tris buffer pH 7.4. Steroid metabolism was then assayed as per section 2.6.2.

2.10 ASSAY OF MITOCHONDRIAL PYRUVATE DEHYDROGENASE ACTIVITY

Mitochondrial pyruvate dehydrogenase activity was assayed by measuring the conversion of ^{14}C -pyruvic acid to $^{14}\text{CO}_2$, as described by Saltiel (1987).

Rat liver mitoplasts (2.5mg) protein were prepared as per section 2.3, and incubated with 0.2mM ATP for 10min at 37°C in the presence of 50 μM CaCl_2 and 50 μM MgCl_2 to convert the enzyme into its inactive phosphorylated form. Following this incubation the solutions were cooled to 4°C and twice washed free of unbound nucleotide by centrifugation and resuspension in 10mM potassium phosphate buffer pH 7.0.

Pyruvate dehydrogenase activity was assayed, in triplicate, by monitoring the conversion of ^{14}C -pyruvic acid to $^{14}\text{CO}_2$ in 1.0ml containing 50 μM coenzyme A, 50 μM co-carboxylase, 50 μM MgCl_2 , 25 μM CaCl_2 , 1mM dithiothrietol, 0.5mM β -NAD and 0.5mM pyruvic acid (3000cpm) in 20mM potassium phosphate buffer pH 7.0. The reaction was started by the addition of this reaction mixture to vessels containing varying amounts of the insulin mediator extract and 2.5mg mitochondrial protein, and allowed to proceed for 5min at 37°C . The reaction was then stopped by the addition of 1ml 2N H_2SO_4 , and the release of $^{14}\text{CO}_2$ was determined by absorption to phenylethylamine-soaked filter paper squares (1cm²) suspended above the reaction system from the rubber stoppers capping the tubes. $^{14}\text{CO}_2$ was allowed to absorb onto the filter paper for 30min at room temperature, after which the radioactivity in the filter paper was counted by liquid

scintillation counting in 3ml Ecoscint liquid scintillant. Pyruvate dehydrogenase activity was expressed as nmole /mg protein /minute.

2.11 CHROMATOGRAPHY OF THE ACID HYDROLYSED INSULIN MEDIATOR

Samples of the original insulin mediator extract were subject to acid-hydrolysis by incubating with 2ml of 10N HCl for 2h at 100°C. Both hydrolysed and unhydrolysed mediator fractions were then spotted onto cellulose thin layer chromatography plates and onto 20x20cm squares of Whatman No. 1 filter paper. Samples of 5% standard solutions of galactose, mannose, glucosamine, galactosamine and inositol were also applied. The chromatograms were then placed in a TLC tank equilibrated with butanol : acetic acid : water (4:1:2) and the solvent front allowed to run about 1cm from the edge in each case. The chromatograms were left to dry overnight at room temperature, and then placed in an iodine vapour tank to locate the components of chromatography. Comparison of the insulin mediator to the standards allowed identification of the bands visualised.

2.12 CALCULATION OF RESULTS AND STATISTICS

Enzyme activities are expressed as pmoles product / minute / million cells or as pmoles product / minute / mg protein \pm standard deviation of triplicate incubations. Absolute values are then expressed as percentages of

relevant controls \pm standard deviation and statistical analysis performed by means of Student's t-test. The level of significance has been set at $p < 0.05$ in each case.

2.13 BUFFERS

1) Calcium-free HBSS (10% stock solution)

KCl.....	2.0g/l
KH ₂ PO ₄ (anhydrous).....	0.3g/l
NaCl.....	40g/l
NaHCO ₃ (anhydrous).....	10.5g/l
Na ₂ HPO ₄ (anhydrous).....	0.238g/l

2) Incubation medium

HBSS.....	100ml (10% stock)
glucose.....	1.0g/l
MgCl ₂ .6H ₂ O.....	0.1g/l
MgSO ₄ .7H ₂ O.....	0.1g/l
CaCl ₂ .6H ₂).....	0.185g/l

3) Sodium acetate buffer

50mM sodium acetate solution adjusted to pH 5 using acetic acid.

4) 100mM Potassium phosphate buffer

66.25ml of 200mM K₂HPO₄

183.75ml of 200mM KH₂PO₄

-made up to 500ml with distilled water and adjust to pH 6.4 using 10N HCl.

5) 0.25M Sucrose-0.1M Tris buffer pH 7.4

132g/l sucrose

12.1g/l Tris base

80ml/l 1N HCl

2.14 MATERIALS LIST

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Androst-4-ene-3,17-dione

Dexametasone-21-acetate

L-thyroxine (sodium salt)

Tris buffers

cyclic AMP

BCL Ltd., Lewes, East Sussex, U.K.

Collagenase

Bovine serum albumin

NADP

Isocitric acid

Isocitrate dehydrogenase

Gibco Ltd., Paisley, Strathclyde, U.K.

Ham's F-10 culture medium

Penicillin/streptomycin

Novo Research Centre, Copenhagen, Denmark

Porcine insulin

LKB, Bromma, Sweden

Ultroser G

KabiVitrum AB, Stockholm, Sweden

Human growth hormone

Kodak, France

X-ray film (X-Omat S)

Developer (D-19)

Fixer (FX-40)

Amersham Int. plc, Aylesbury, Bucks., U.K.

[4- ^{14}C]-androst-4-ene-3,17-dione

[1- ^{14}C]-pyruvic acid

Adenosine-3'5'-cyclic phosphoric acid 2'-O-succinyl-3 [125~~I~~]

iodotyrosine methyl ester [^{125}I]-cAMP

All other chemicals were of the highest grade available commercially.

RESULTS

3.0 RESULTS

The assay used allows the measurement of the 6 β -hydroxylase, 7 α -hydroxylase, 16 α -hydroxylase, 3 α / β -oxosteroid oxidoreductase, 17-oxosteroid oxidoreductase and 5 α -reductase activities. 3 α / β -oxosteroid oxidoreductase, however, does not metabolise the parent compound (Figure 3), but rather the metabolite subsequent to 5 α -reduction of androstenedione. Only primary metabolism of the substrate was studied, and so the 3 α / β -oxosteroid oxidoreductase product was measured with unchanged 5 α -androstane-3,17-dione so as to determine total 5 α -reductase activity.

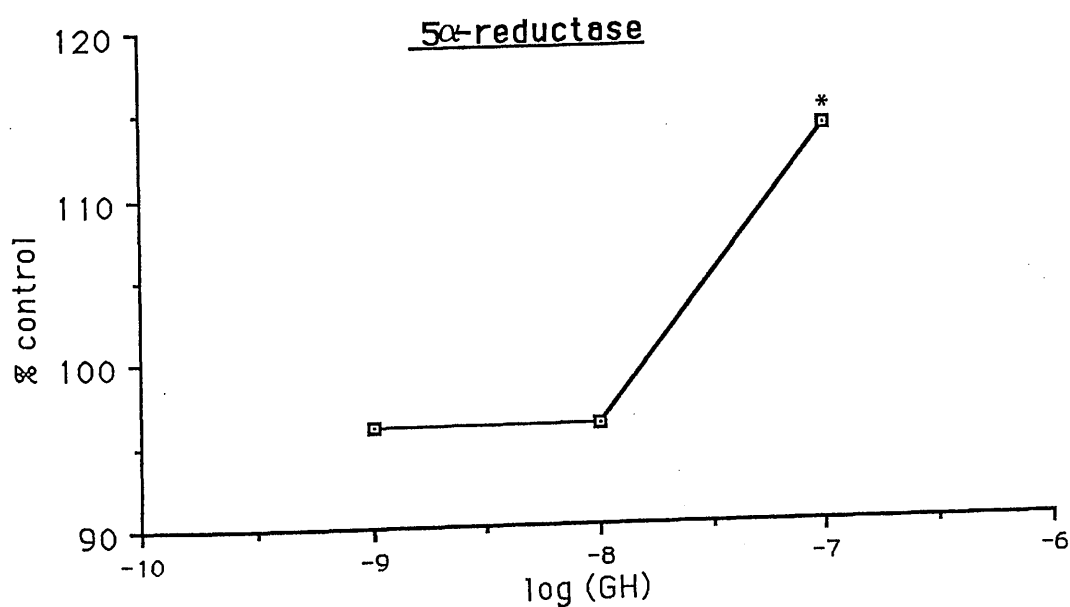
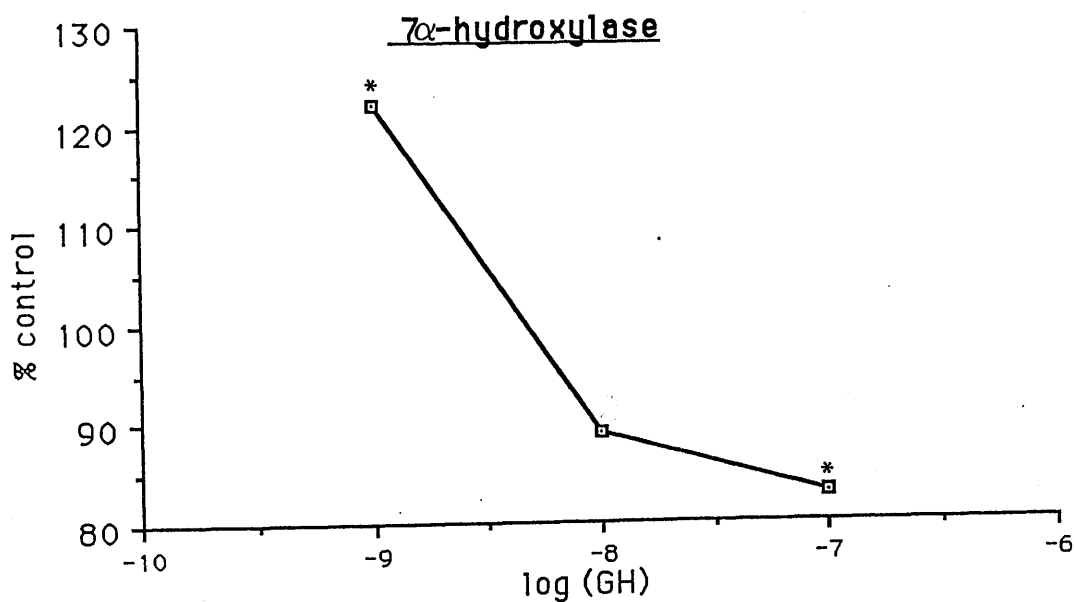
The two female-specific activities (7 α -hydroxylase and 5 α -reductase) are shown in the first graph, and the male-specific activities (6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase are shown in the second graph in each case. Results are illustrated as percentages of relevant controls and tabulated as absolute values in pmoles product/minute/million cells \pm standard deviation. Error bars have been omitted from the graphs for reasons of clarity, but significant points have been asterisked.

3.1 GROWTH HORMONE

3.1.1 DOSE-DEPENDENT EFFECTS OF GROWTH HORMONE

Hepatocytes were preincubated with 10^{-9} to 10^{-7} M growth hormone for 30 minutes prior to assaying for steroid-metabolising activity (Table 5). As compared to

FIGURE 6 : Dose-response effects of 30 minute preincubation with 10^{-9}M - 10^{-7}M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 5



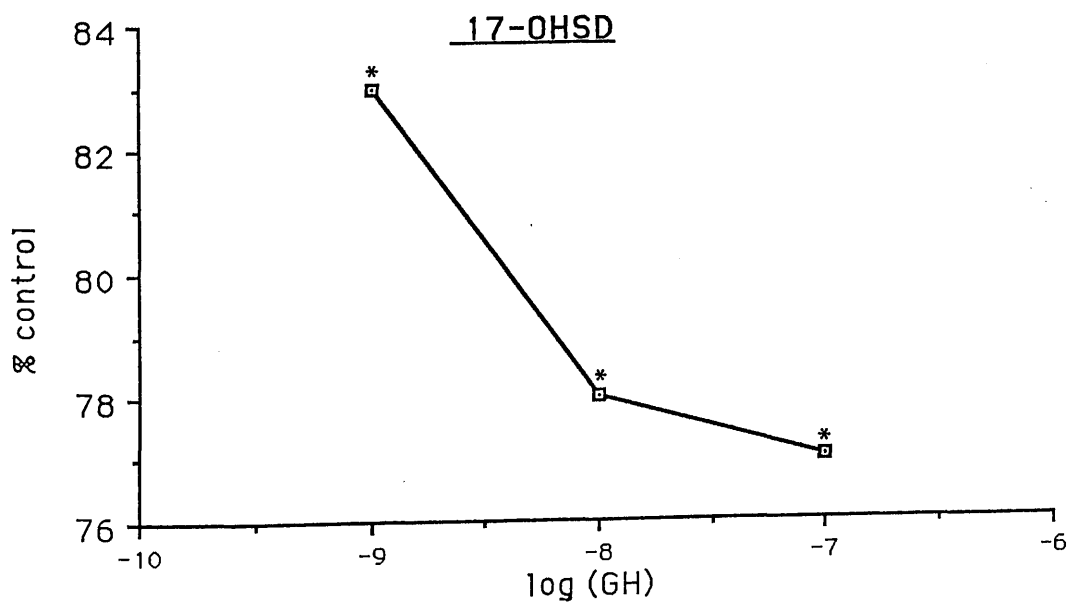
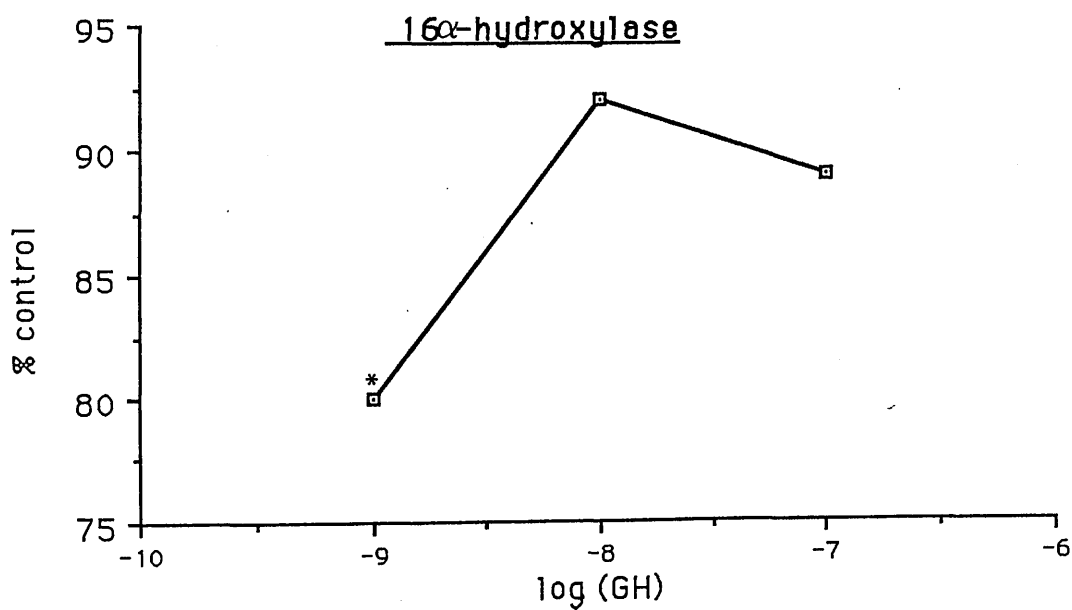
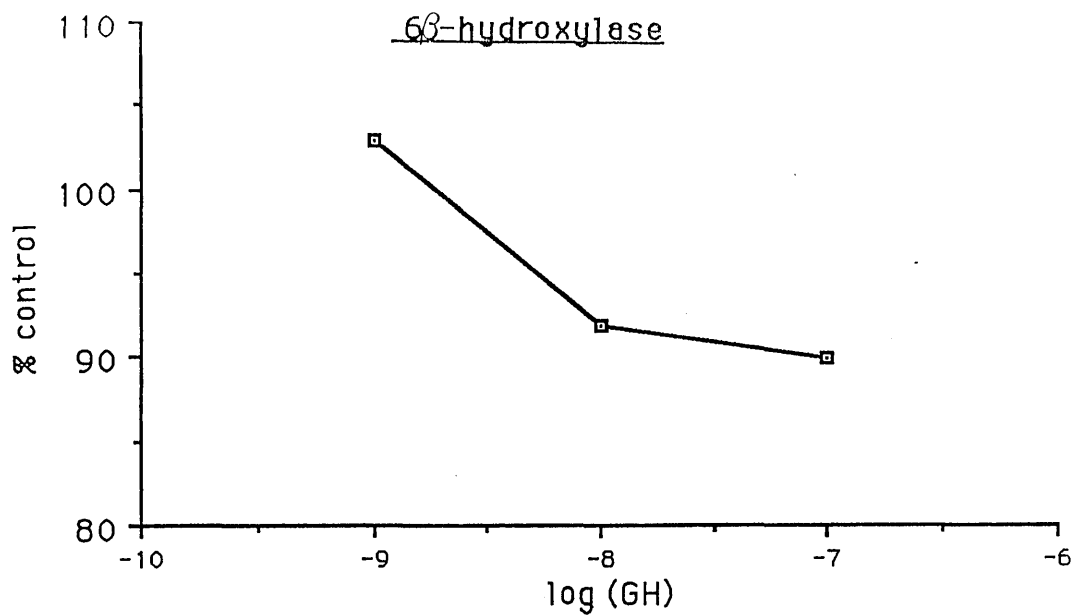


TABLE 5 : Dose-response effects of 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities after 30 minute preincubation with 10⁻⁹M-10⁻⁷M GROWTH HORMONE.

Enzyme activities (pmoles/minute/million cells)					
TREATMENT	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
Control (A)	36 \pm 4	38 \pm 4	36 \pm 4	60 \pm 7	100 \pm 2
10 ⁻⁹ M GH (B)	44 \pm 2*	39 \pm 6	29 \pm 3*	50 \pm 1*	96 \pm 8
10 ⁻⁸ M GH (C)	32 \pm 2	35 \pm 4	33 \pm 3	47 \pm 1*	96 \pm 6
10 ⁻⁷ M GH (D)	30 \pm 2*	34 \pm 3	32 \pm 5	46 \pm 2*	114 \pm 8*

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant controls.

Duncan's Multiple Range Test :

7 α -OHase	<u>D C A B</u>
6 β -OHase	<u>D C A B</u>
16 α -OHase	<u>B D C A</u>
17-OHSD	<u>D C B A</u>
5 α -red	<u>B C A D</u>

relevant control values, incubation with 10^{-9} M growth hormone significantly increased 7 α -hydroxylase (Figure 6A), but markedly decreased enzyme activity at the higher concentrations of growth hormone tested. The effects of growth hormone on 5 α -reductase activity were not significant (Figure 6B), except in the case at 10^{-7} M where a 14% increase was observed. Growth hormone had no significant effects on the male-specific enzyme activities 6 β -hydroxylase (Figure 6C) and 16 α -hydroxylase (Figure 6D), but a significant reduction in 17-oxosteroid oxidoreductase activity was observed at all concentrations of growth hormone tested (Figure 6E).

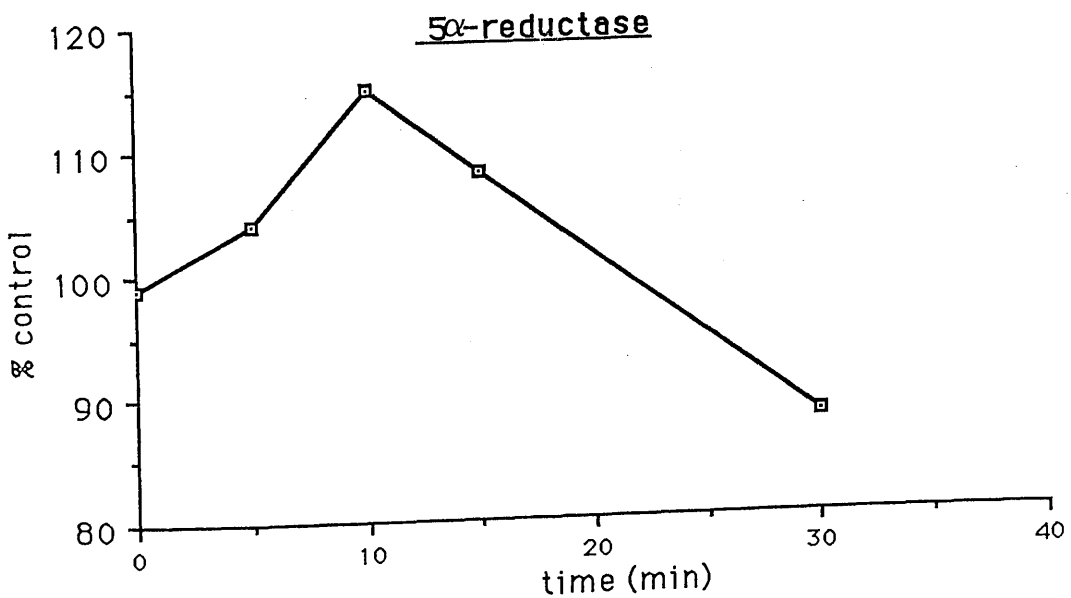
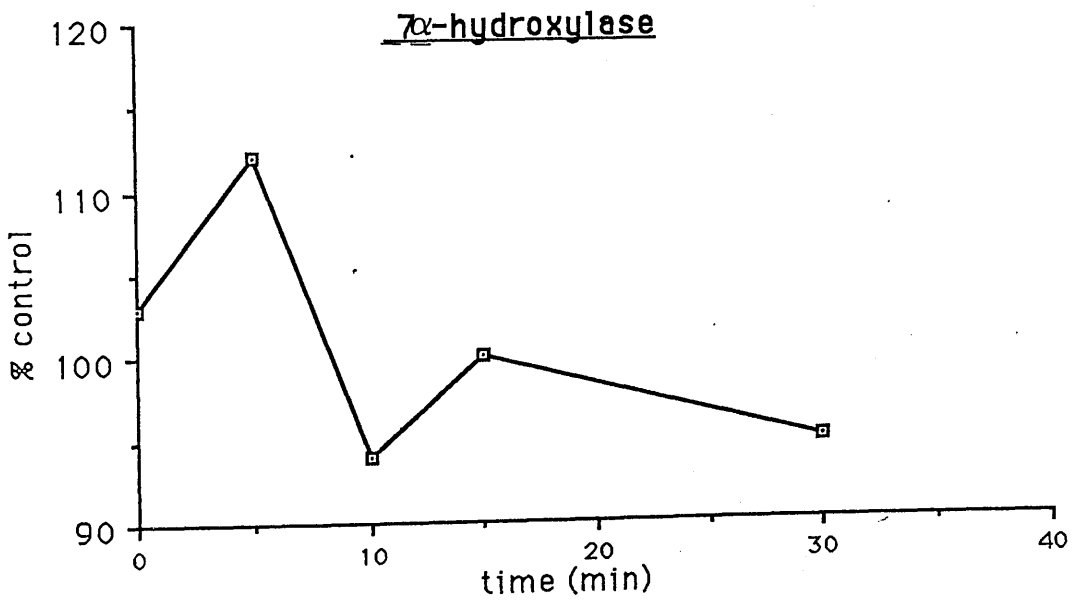
3.1.2 TIME COURSE OF THE EFFECTS OF 10^{-9} M GROWTH HORMONE

Hepatocytes were preincubated with 10^{-9} M growth hormone for 0,5,10,15 and 30 minutes (Table 6). Growth hormone had no significant effects on 7 α -hydroxylase (Figure 7A) and 5 α -reductase (Figure 7B) activities, although a slight increase was observed at the earlier incubation times. No significant effects of incubating with growth hormone were observed on 6 β -hydroxylase activity (Figure 7C), except at 15 minutes of incubation where a significant decrease was seen. Growth hormone significantly decreased both 16 α -hydroxylase (Figure 7D) and 17-oxosteroid oxidoreductase activities (Figure 7E) between 10 and 30 minutes of incubation.

Hepatocytes were preincubated with 10^{-9} M growth hormone

FIGURE 7 : Time course of the effects of preincubation with 10^{-9} M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 6



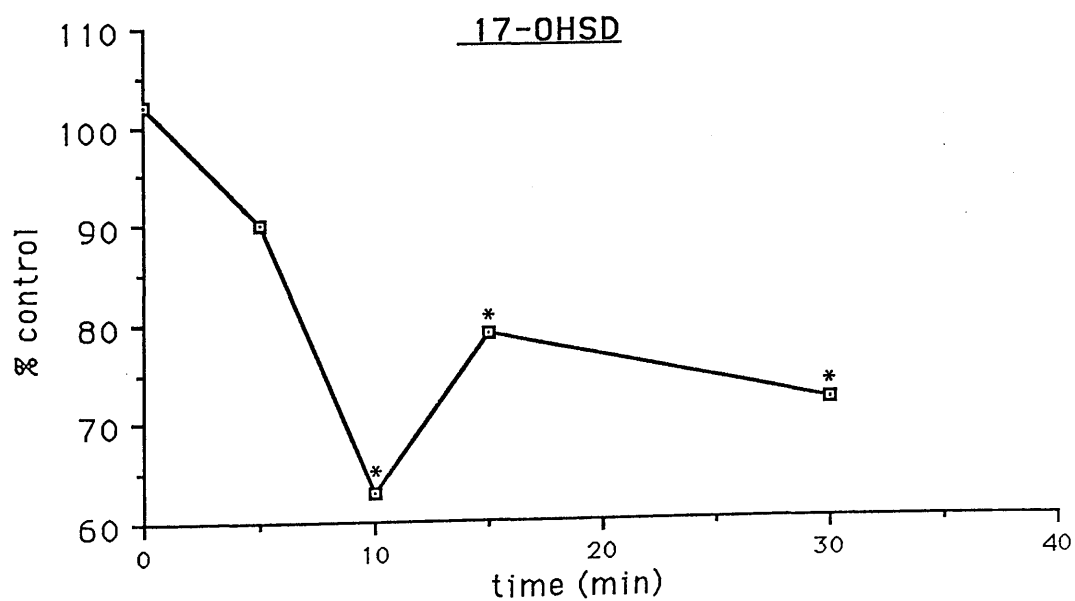
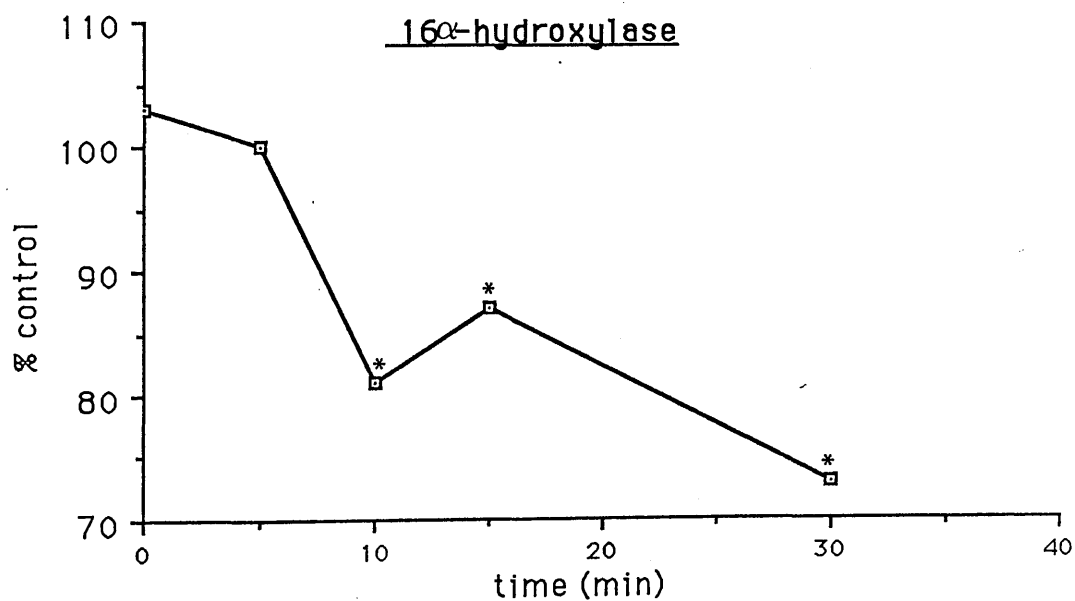
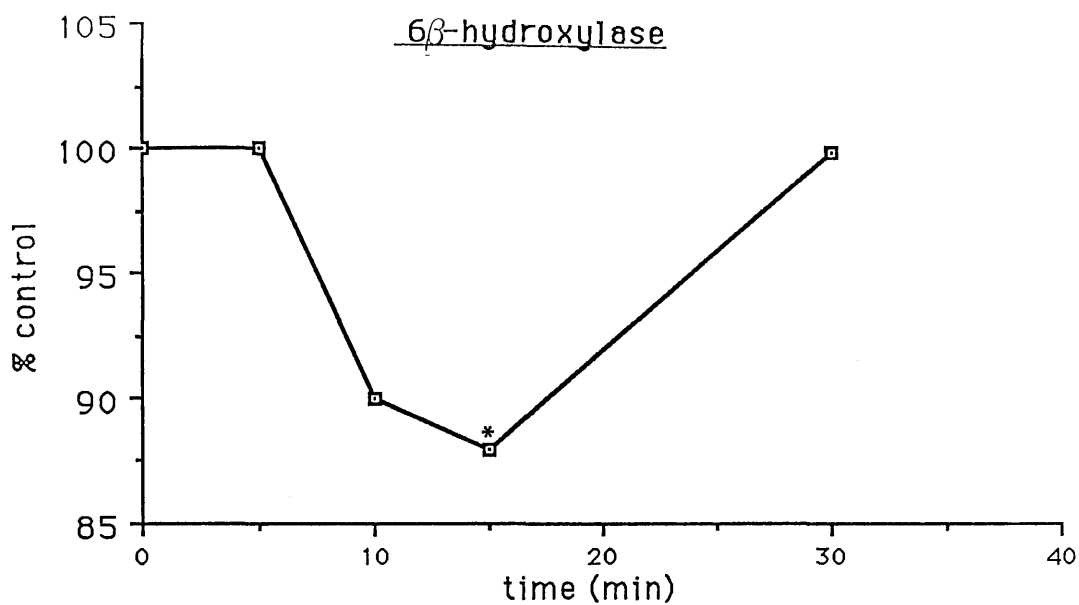


TABLE 6 : Effects of preincubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 7α -, 6β -, 16α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5α -reductase activities.

TIME(min)	Enzyme activities (pmoles/minute/million cells)				
	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
0C	34 \pm 5	36 \pm 5	34 \pm 6	58 \pm 7	106 \pm 9
0GH	35 \pm 3	36 \pm 2	35 \pm 2	59 \pm 5	105 \pm 7
5C	34 \pm 8	34 \pm 6	30 \pm 7	58 \pm 8	100 \pm 10
5GH	38 \pm 7	34 \pm 4	30 \pm 1	52 \pm 8	104 \pm 3
10C	34 \pm 3	38 \pm 8	32 \pm 1	38 \pm 1	92 \pm 5
10GH	32 \pm 2	34 \pm 3	26 \pm 3*	44 \pm 1*	106 \pm 16
15C	30 \pm 2	34 \pm 1	30 \pm 2	58 \pm 4	98 \pm 4
15GH	30 \pm 4	30 \pm 2*	26 \pm 2*	46 \pm 2*	106 \pm 8
30C	38 \pm 2	38 \pm 1	44 \pm 4	78 \pm 3	118 \pm 12
30GH	36 \pm 7	38 \pm 1	32 \pm 6*	56 \pm 8*	118 \pm 12

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values (C=control).

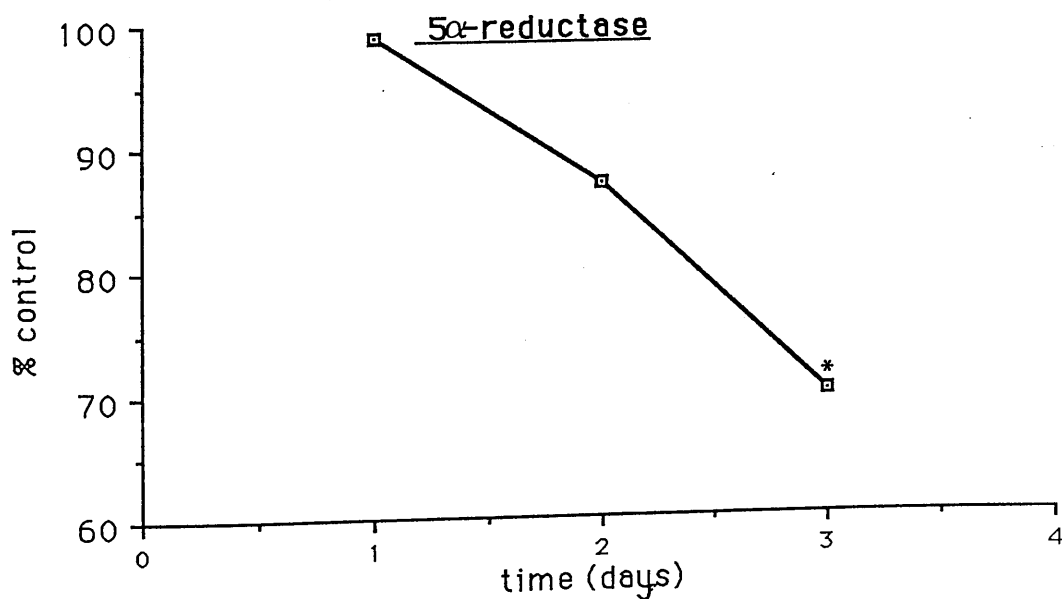
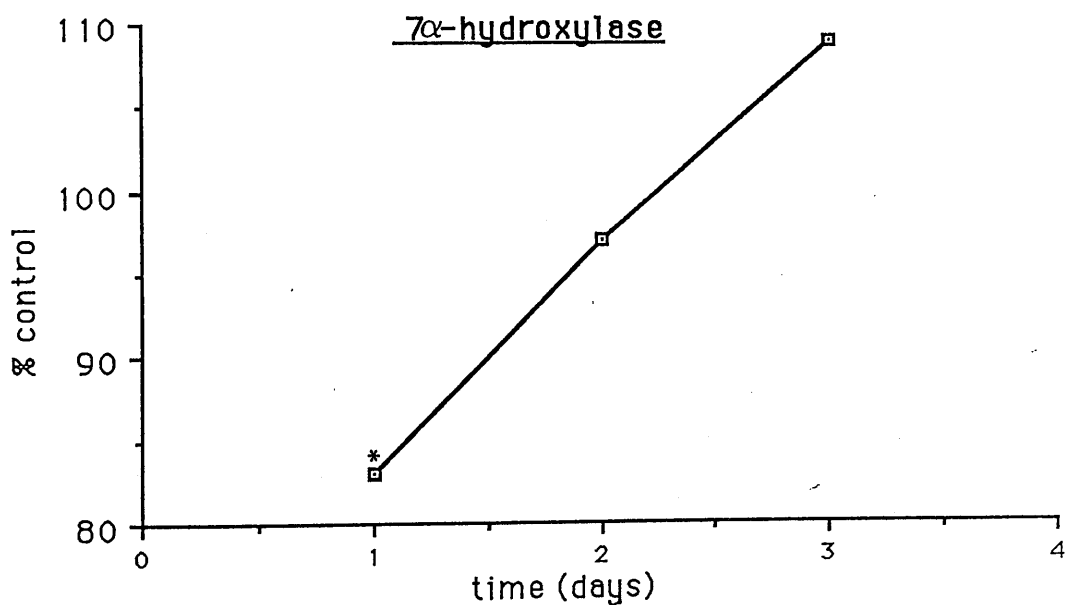
over 24, 48 and 72 hours (Table 7) as described in Section 2.5.2. Figure 8A shows that growth hormone significantly decreased 7 α -hydroxylase activity after 24h of incubation, after which enzyme activity returned to basal levels. No significant effects of growth hormone were seen on 5 α -reductase activity (Figure 8B), except at 72h where enzyme activity was decreased to 70% of control. No changes in enzyme activity were observed with 6 β -hydroxylase (Figure 8C) and 16 α -hydroxylase activities (Figure 8D), whilst a significant increase in 17-oxosteroid oxidoreductase (Figure 8E) activity was seen at 24 and 48h of incubation, an effect that was lost by 72h.

3.1.3 TIME COURSE OF THE EFFECTS OF 10⁻⁸M GROWTH HORMONE

Incubation of hepatocytes with 10⁻⁸M growth hormone (Table 8) exhibited no significant changes in 7 α -hydroxylase (Figure 9A), 6 β -hydroxylase (Figure 9C) or 17-oxosteroid oxidoreductase activities (Figure 9E) throughout the 30 minutes of incubation. 5 α -reductase activity (Figure 9B) was significantly increased after 5 minutes of incubation, and a significant decrease was observed in 16 α -hydroxylase activity (Figure 9D) at 15 minutes of incubation.

Thus although some significant results were obtained by incubating hepatocytes with growth hormone, these effects were, firstly, not consistent over all experiments and, secondly the results obtained are not typical of those obtained by in vivo infusion of growth hormone (Mode et al

FIGURE 8 : Effects of preincubation with 10^{-9} M GROWTH HORMONE over 24, 48 and 72 hours on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 7



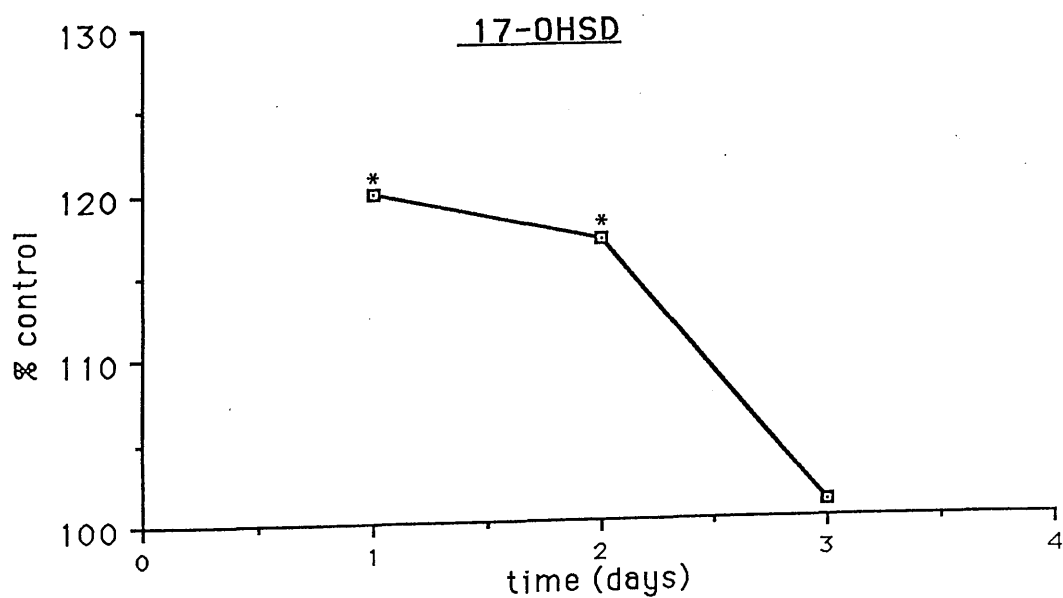
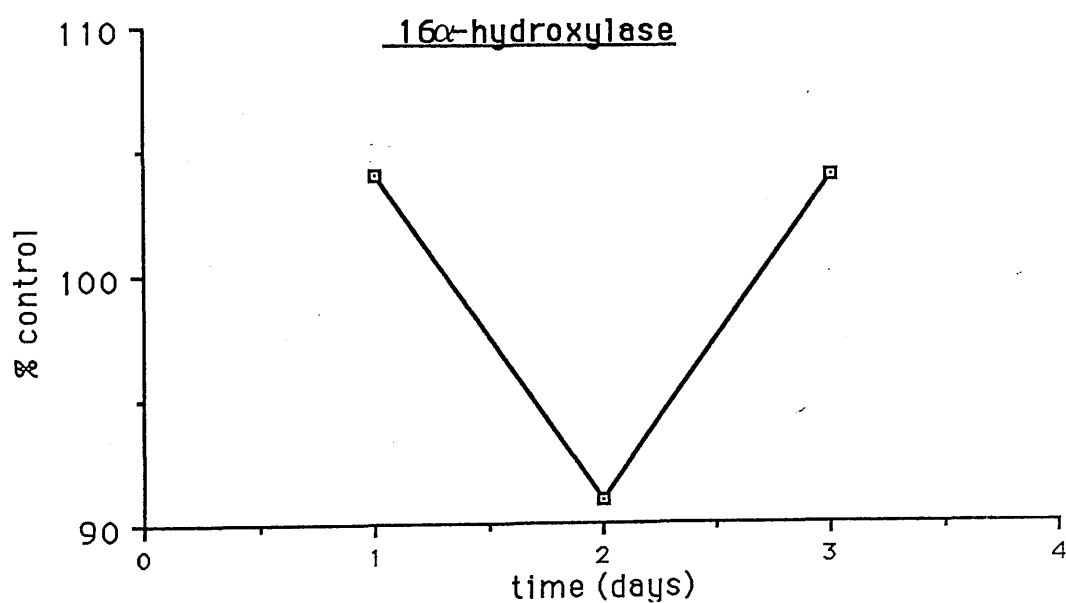
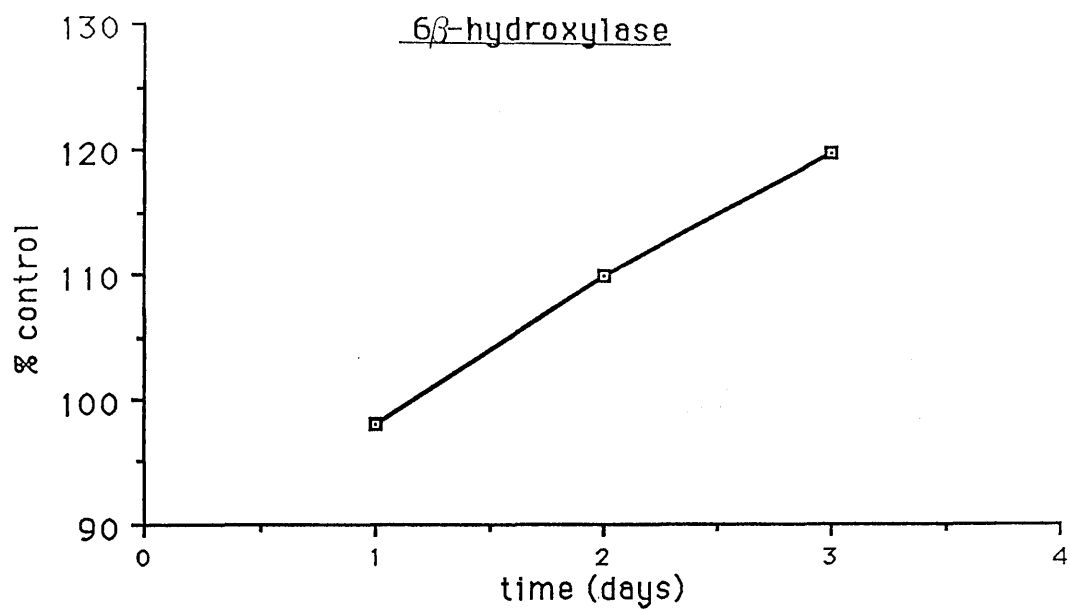


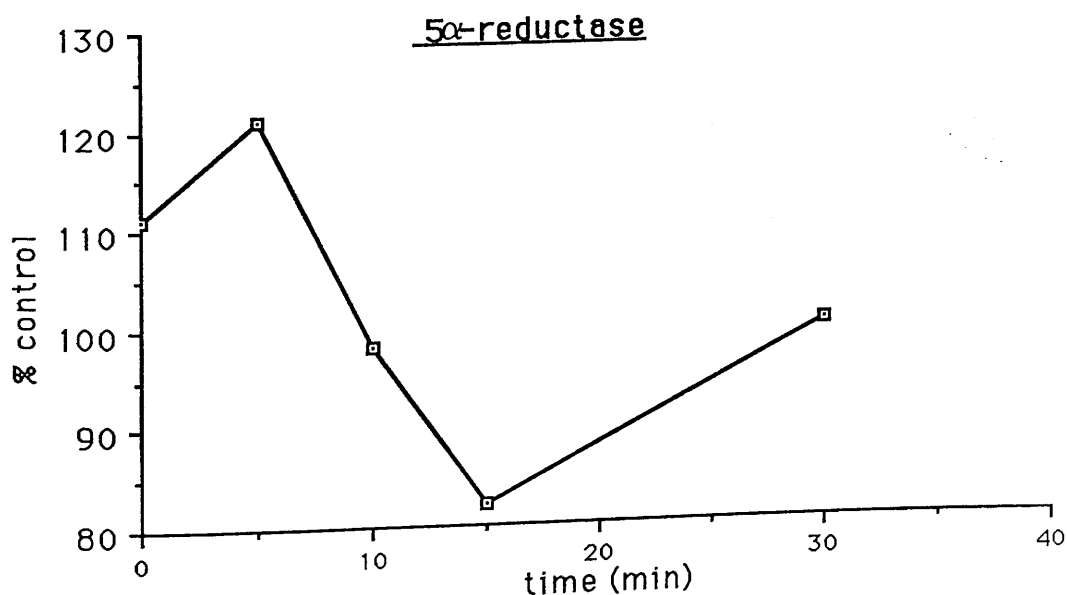
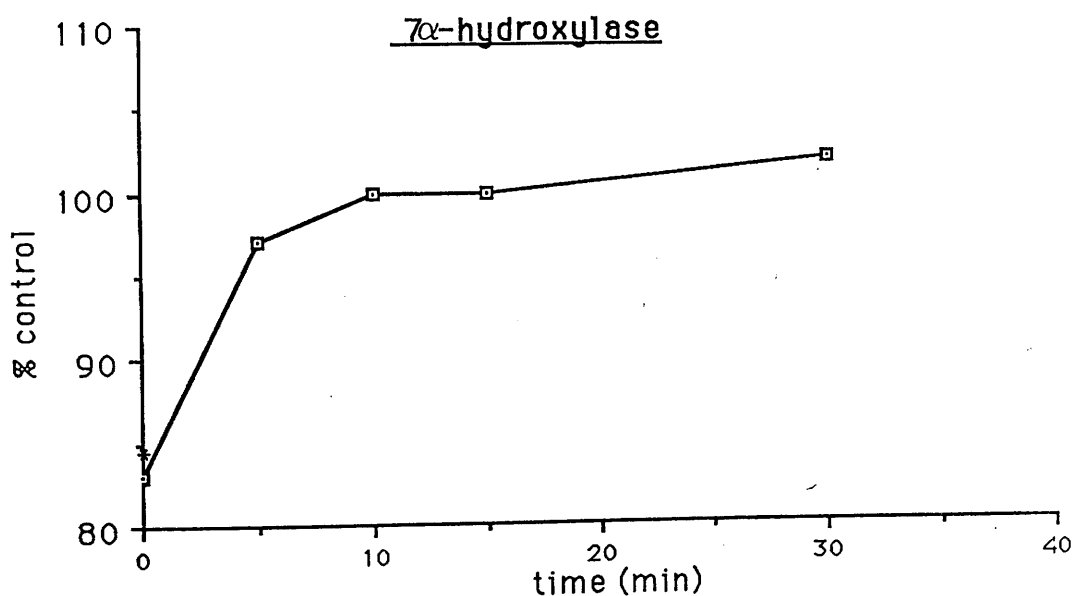
TABLE 7 : Effects of preincubation with 10^{-9} M GROWTH HORMONE over 24,48 and 72h on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

TIME (hrs)	Enzyme activities (pmoles/minute/million cells)				
	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
24h C	28 \pm 1	30 \pm 1	28 \pm 3	39 \pm 2	61 \pm 2
24h GH	23 \pm 2*	29 \pm 3	29 \pm 1	47 \pm 3*	60 \pm 1
48h C	23 \pm 2	22 \pm 3	26 \pm 3	42 \pm 4	62 \pm 6
48h GH	22 \pm 2	25 \pm 4	24 \pm 2	49 \pm 3*	54 \pm 6
72h C	17 \pm 3	24 \pm 3	27 \pm 3	52 \pm 1	54 \pm 2
72h GH	20 \pm 1	28 \pm 4	28 \pm 1	50 \pm 2	37 \pm 4*

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values (C=control).

FIGURE 9 : Time course of the effects of preincubation with 10^{-8} M GROWTH HORMONE on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 8



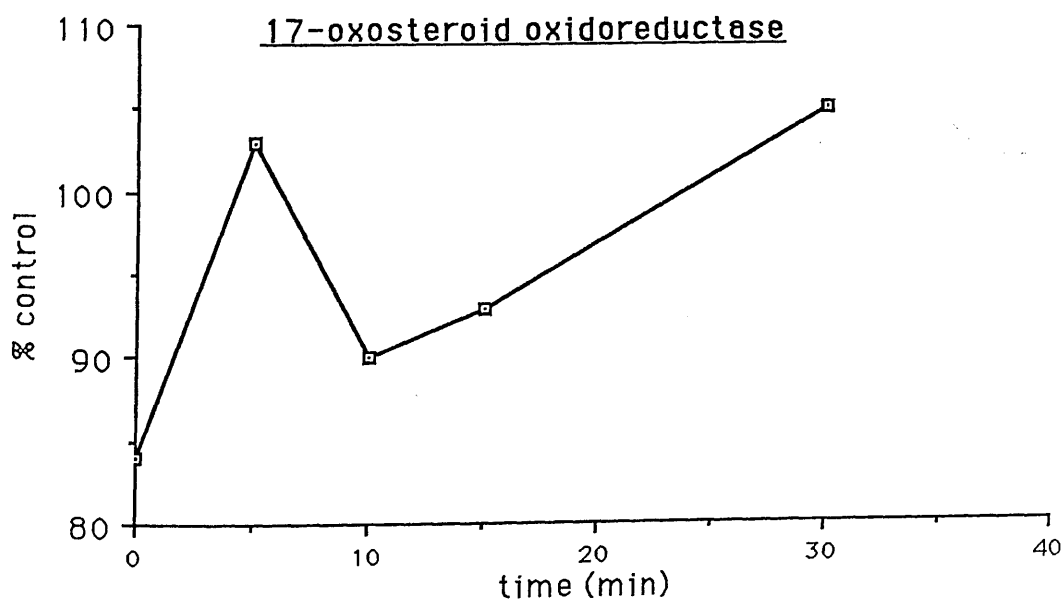
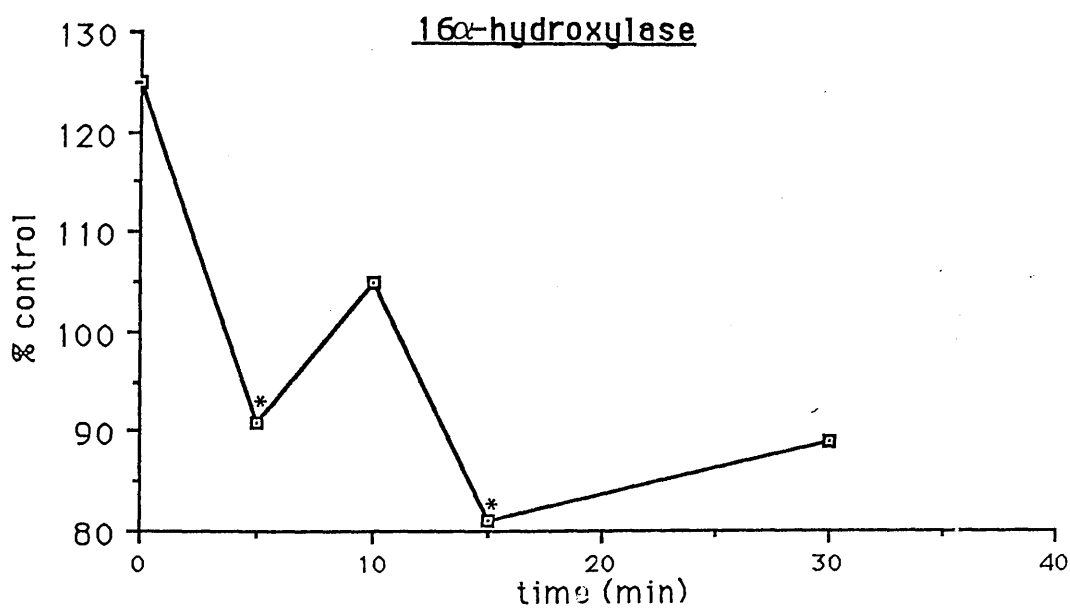
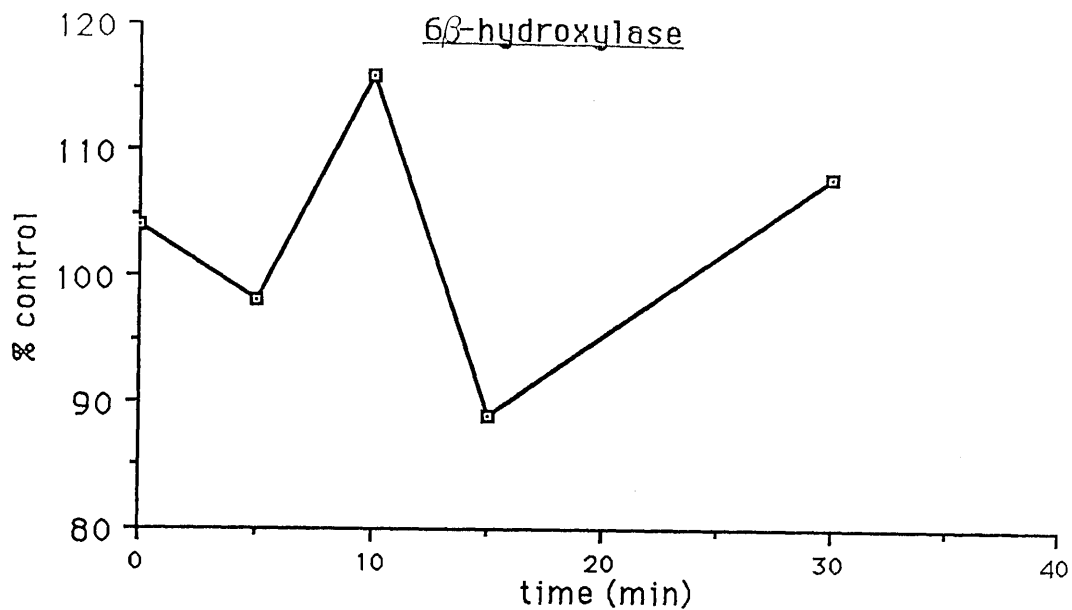


TABLE 8 : Effects of 30 minute preincubation with 10^{-8} M GROWTH HORMONE on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

Enzyme activities (pmoles/minute/million cells)					
TIME (min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
0C	64 \pm 18	58 \pm 10	44 \pm 15	61 \pm 9	118 \pm 7
0GH	54 \pm 5	60 \pm 6	56 \pm 2	51 \pm 6	132 \pm 14
5C	52 \pm 18	62 \pm 6	54 \pm 3	45 \pm 7	115 \pm 6
5GH	54 \pm 4	60 \pm 4	48 \pm 2*	46 \pm 4	139 \pm 3
10C	44 \pm 6	47 \pm 4	47 \pm 7	48 \pm 5	100 \pm 2
10GH	44 \pm 4	54 \pm 7	50 \pm 2	42 \pm 5	98 \pm 8
15C	56 \pm 2	58 \pm 2	64 \pm 6	48 \pm 2	125 \pm 9
15GH	56 \pm 6	53 \pm 9	52 \pm 4*	45 \pm 4	102 \pm 5
30C	44 \pm 6	50 \pm 10	48 \pm 9	38 \pm 2	95 \pm 11
30GH	46 \pm 4	54 \pm 10	42 \pm 6	40 \pm 6	146 \pm 6

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values (C=control).

1982), as will be discussed in a later section.

3.1.4 EFFECTS OF GROWTH HORMONE ON INTRACELLULAR CYCLIC AMP

Table 9 shows that incubation of hepatocytes with 10^{-9} M growth hormone over 0-30 minutes (Figure 10) and over 24, 48 and 72 hours (Figure 11) had no significant effects on intracellular cyclic AMP levels.

3.1.5 DOSE-DEPENDENT EFFECTS OF INSULIN-LIKE GROWTH FACTOR 1

Incubation of hepatocytes with 10^{-10} to 10^{-8} M insulin-like growth factor 1 (IGF-1) for 30 minutes (Table 10) showed dose-dependent increases in all enzyme activities. Maximum effects of IGF-1 on 7 α -hydroxylase activity (Figure 12A) were seen upon incubation with 10^{-9} M IGF-1. 5 α -reductase (Figure 12B), 6 β -hydroxylase (Figure 12C), 16 α -hydroxylase (Figure 12D) and 17-oxosteroid oxidoreductase (Figure 12E) activities all exhibited dose-dependent effects with maxima at 10^{-8} M IGF-1.

FIGURE 10 : Intracellular cyclic AMP content in hepatocytes treated with 10^{-9} M GROWTH HORMONE over 0-120 minutes. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 9

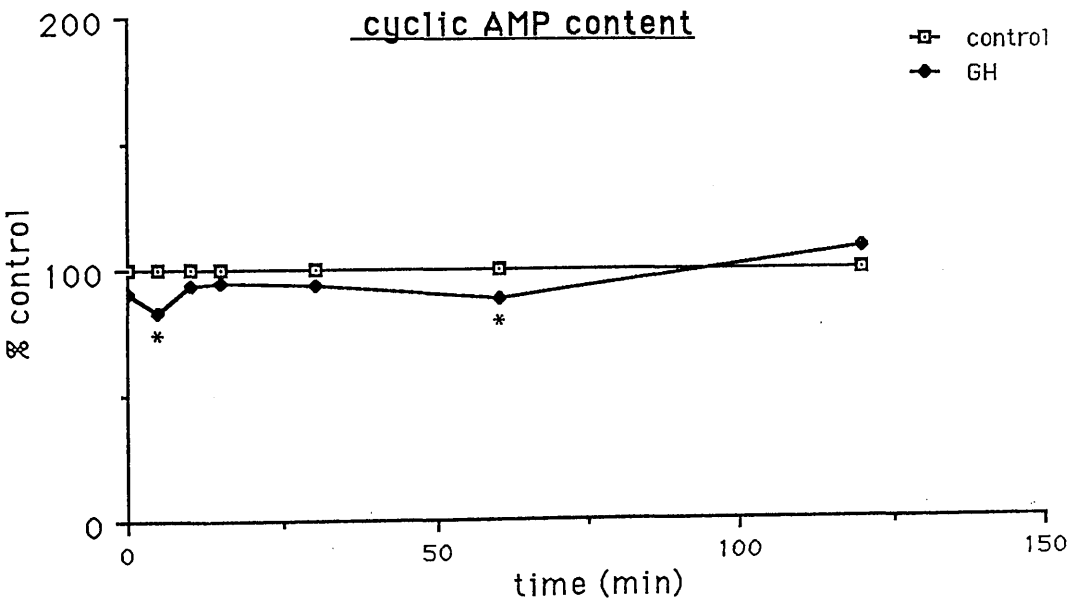


FIGURE 11 : Intracellular cyclic AMP content in hepatocytes treated with 10^{-9} M GROWTH HORMONE over 24, 48 and 72 hours. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 9

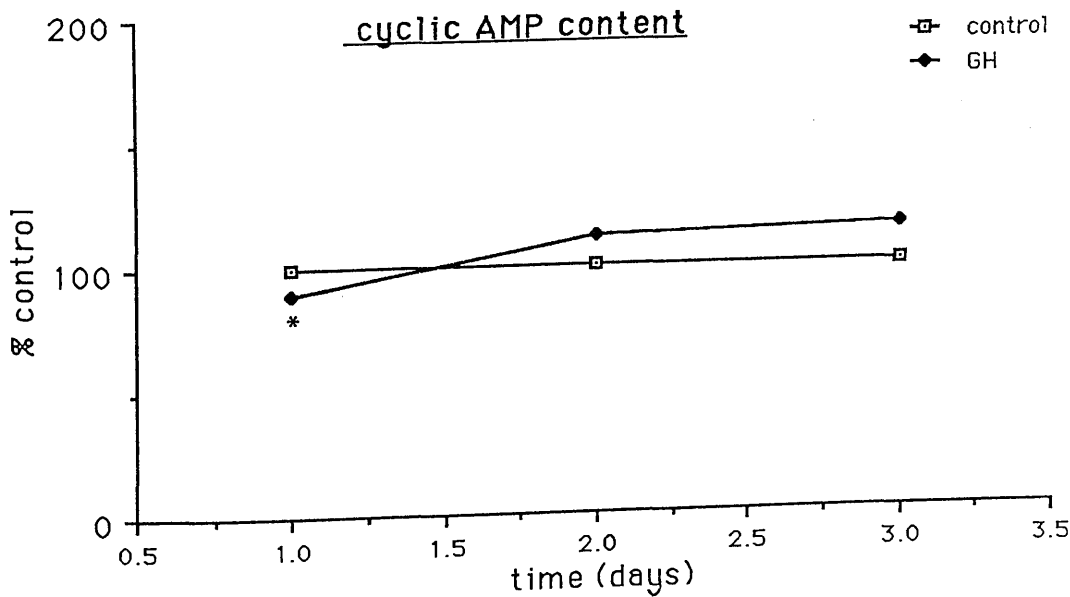
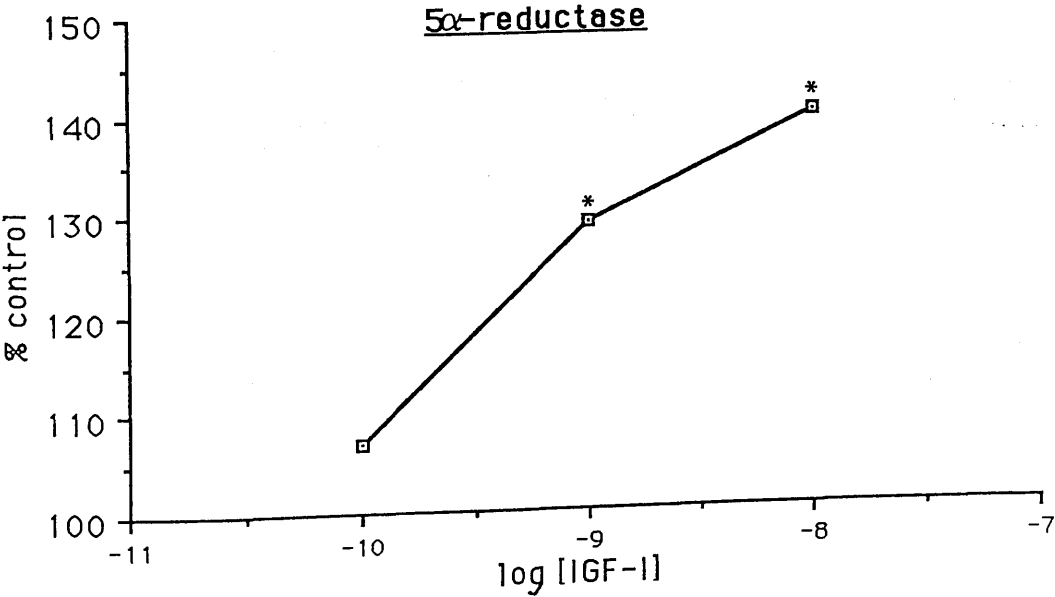
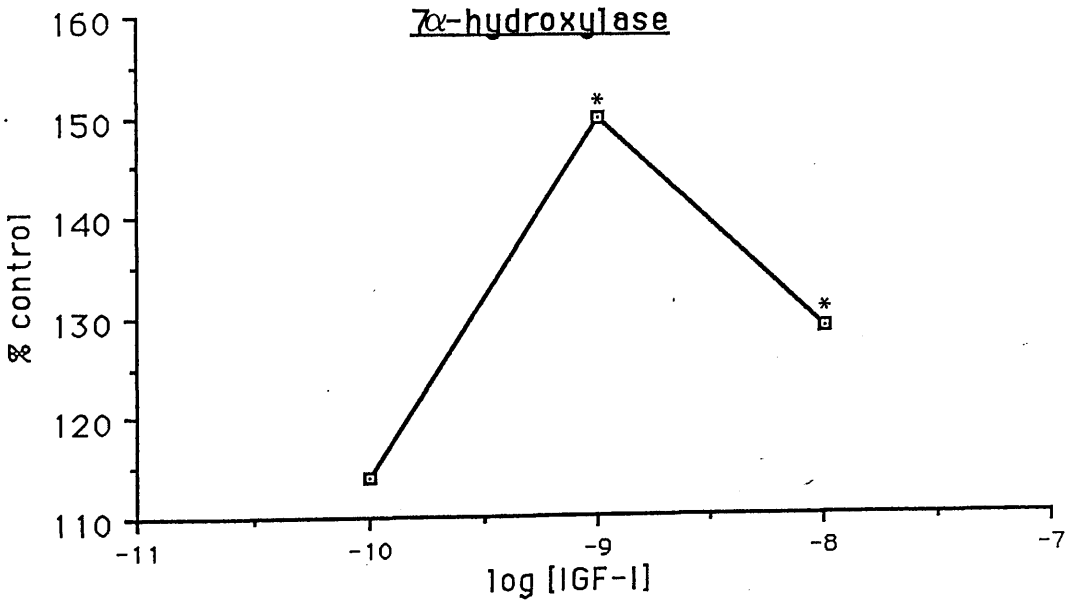


TABLE 9 : Effects of preincubation with 10^{-9} M GROWTH HORMONE on intracellular cyclic AMP content.

<u>TIME</u>	<u>CONTROL</u>	<u>10^{-9}M GH</u>
0min	2.0 \pm 0.23	1.8 \pm 0.21
5min	1.8 \pm 0.14	1.5 \pm 0.13*
10min	1.8 \pm 0.16	1.7 \pm 0.18
15min	1.9 \pm 0.21	1.8 \pm 0.24
30min	1.7 \pm 0.18	1.6 \pm 0.08
60min	1.6 \pm 0.09	1.4 \pm 0.12*
120min	1.2 \pm 0.10	1.3 \pm 0.15
24hr	0.84 \pm 0.06	0.53 \pm 0.05*
48hr	0.84 \pm 0.07	1.00 \pm 0.14
72hr	0.74 \pm 0.10	0.89 \pm 0.09

Results are expressed as mean \pm s.d. of cyclic AMP levels in pmoles/million cells (N=3), and plotted as percentages of relevant control values.

FIGURE 12 : Effects of 30 minute preincubation with 10^{-10}M - 10^{-8}M INSULIN-LIKE GROWTH FACTOR 1 on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 10



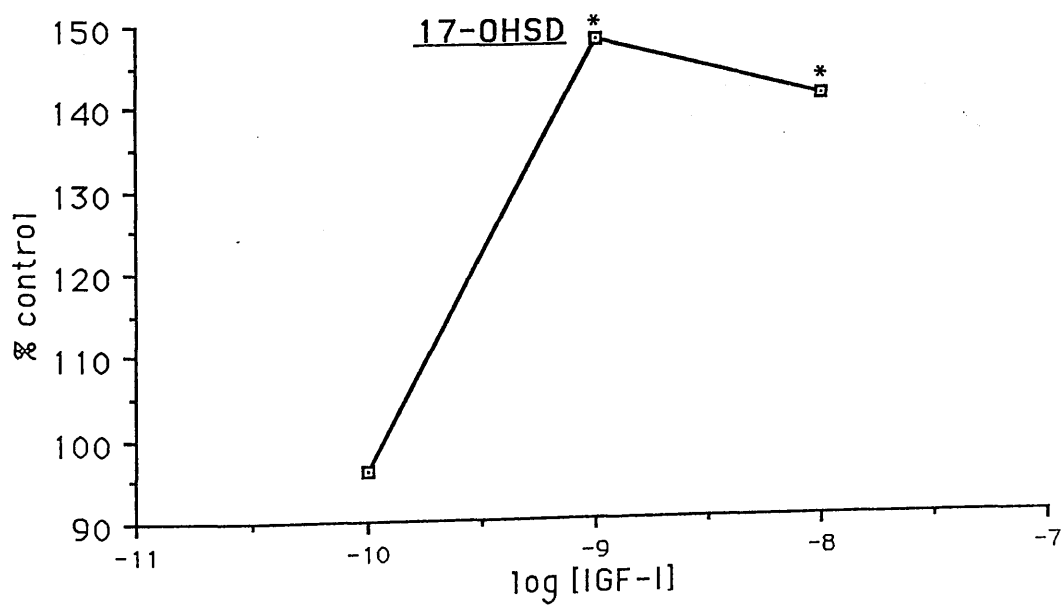
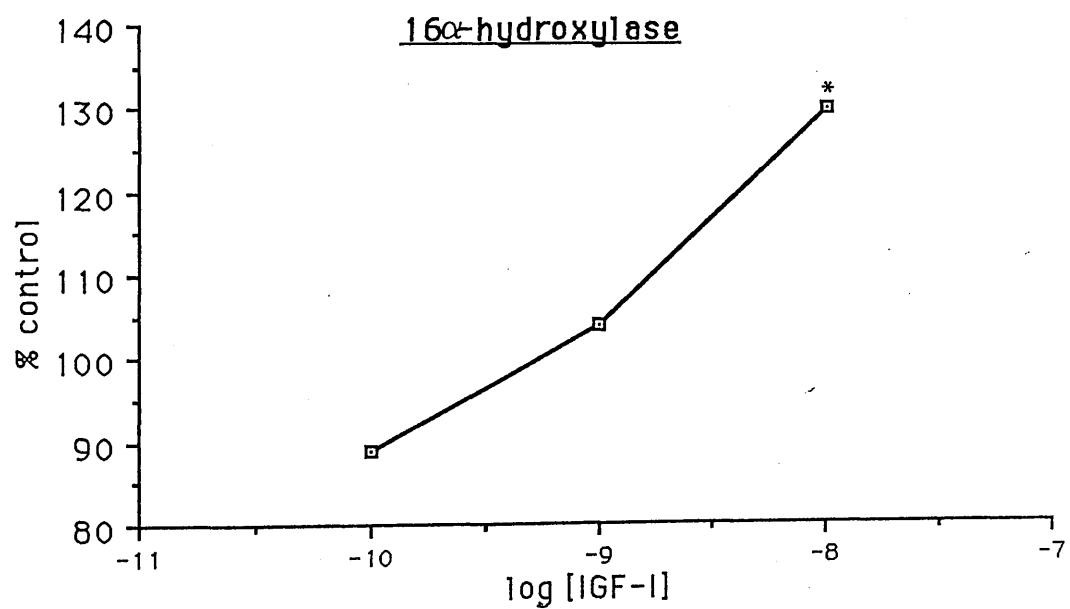
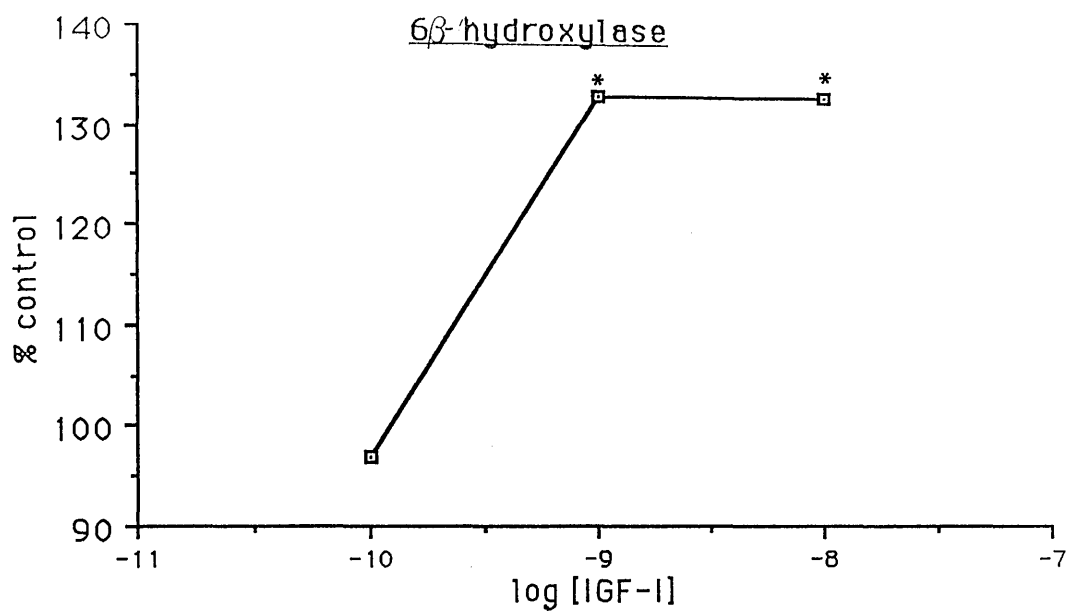


TABLE 10 : Effects of 30 minute preincubation with 10⁻¹⁰M-10⁻⁸M IGF-1 on 7α-, 6β-, 16α-hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5α-reductase activities.

<u>Enzyme activities (pmoles/minute/million cells)</u>					
<u>TREATMENT</u>	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
Control (A)	42±3	45±2	40±3	81±4	126±3
10 ⁻¹⁰ M (B)	48±4	44±2	36±2	78±5	120±7
10 ⁻⁹ M (C)	63±6*	60±3*	42±2	120±8*	152±9*
10 ⁻⁸ M (D)	54±3*	60±5*	52±3*	114±7*	177±6*

Results are expressed as mean ± s.d. (N=3); *=P<0.05 as compared to relevant controls.

Duncan's Multiple Range Test :

7α-OHase	<u>A</u>	<u>B</u>	<u>D</u>	<u>C</u>
6β-OHase	<u>B</u>	<u>A</u>	<u>C</u>	<u>D</u>
16α-OHase	<u>B</u>	<u>A</u>	<u>C</u>	<u>D</u>
17-OHSD	<u>B</u>	<u>A</u>	<u>D</u>	<u>C</u>
5α-red	<u>B</u>	<u>A</u>	<u>C</u>	<u>D</u>

3.2 INSULIN

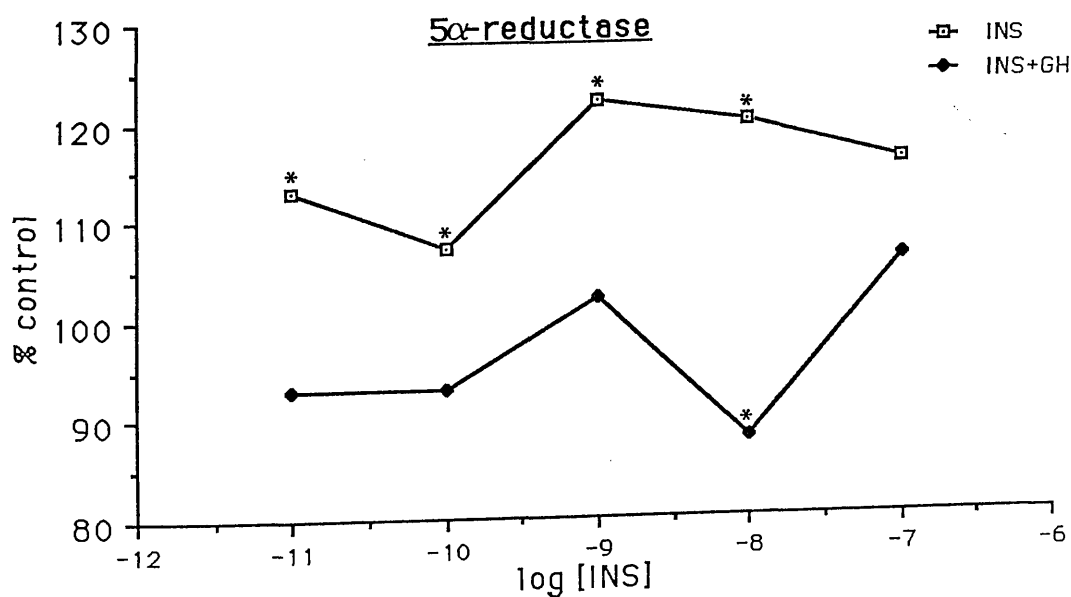
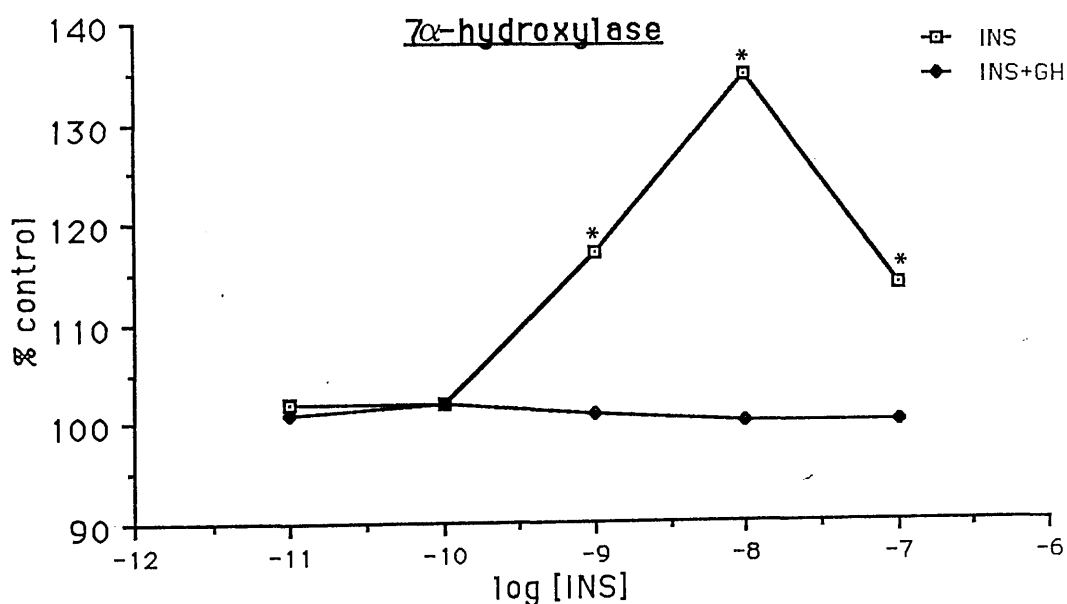
3.2.1 THE EFFECTS OF INSULIN AND THE INTERACTION OF INSULIN AND 10^{-9} M GROWTH HORMONE ON ANDROST-4-ENE-3,17-DIONE METABOLISM

Hepatocytes were preincubated for 30 minutes with 10^{-11} to 10^{-7} M insulin in the presence and absence of 10^{-9} M growth hormone. Incubation of hepatocytes with insulin gave a dose-dependent stimulation of androst-4-ene-3,17-dione metabolism (Table 11A). 7 α -hydroxylase (Figure 13A) and 16 α -hydroxylase (Figure 13D) activities showed maximum stimulation at 10^{-8} M insulin. Maximum activities for 5 α -reductase (Figure 13B), 6 β -hydroxylase (Figure 13C) and 17-oxosteroid oxidoreductase (Figure 13E) were observed with 10^{-9} M insulin. Thus insulin appears to be a non-specific stimulatory agent of steroid metabolism in that both male-and female-specific enzyme activities are affected in a similar fashion, as has been shown previously (Hussin and Skett 1987).

Incubation of hepatocytes with insulin and 10^{-9} M growth hormone resulted in a marked decrease in enzyme activity as compared to the response with insulin alone (Table 11B). This antagonism of the insulin response by growth hormone was more apparent at physiological concentrations of insulin, as is illustrated in Figure 13, and showed insignificant changes in enzyme activity with 10^{-7} M insulin.

These results therefore indicate that, although growth hormone exhibited consistent and significant effects in

FIGURE 13 : Dose-dependent effects of 30 minute preincubation with 10^{-11}M - 10^{-7}M INSULIN in the presence and absence of 10^{-9}M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast = P < 0.05$ and $N = 3$. Absolute data is given in Table 11



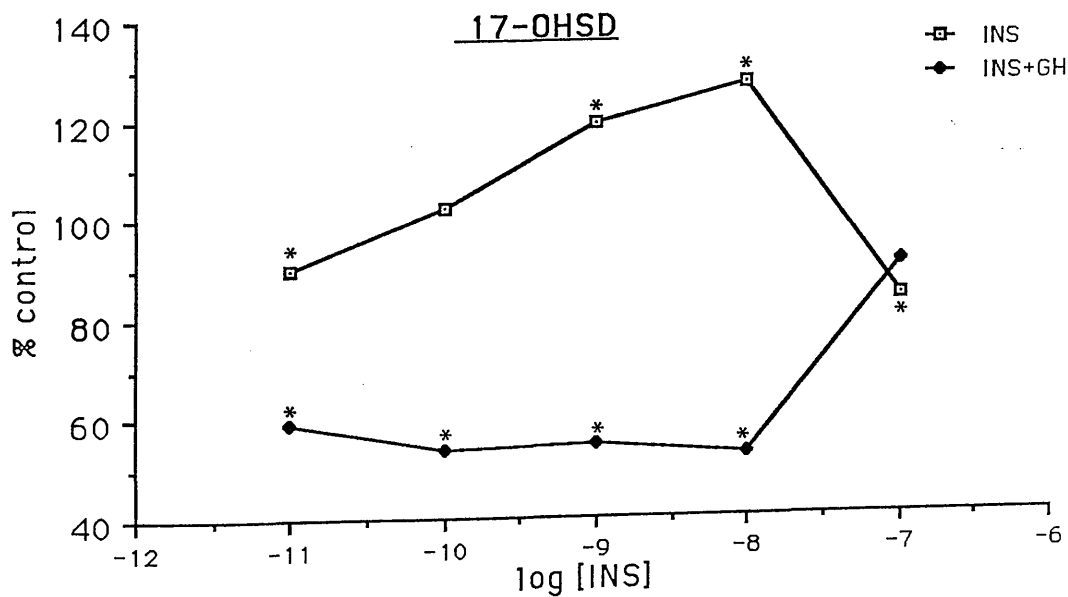
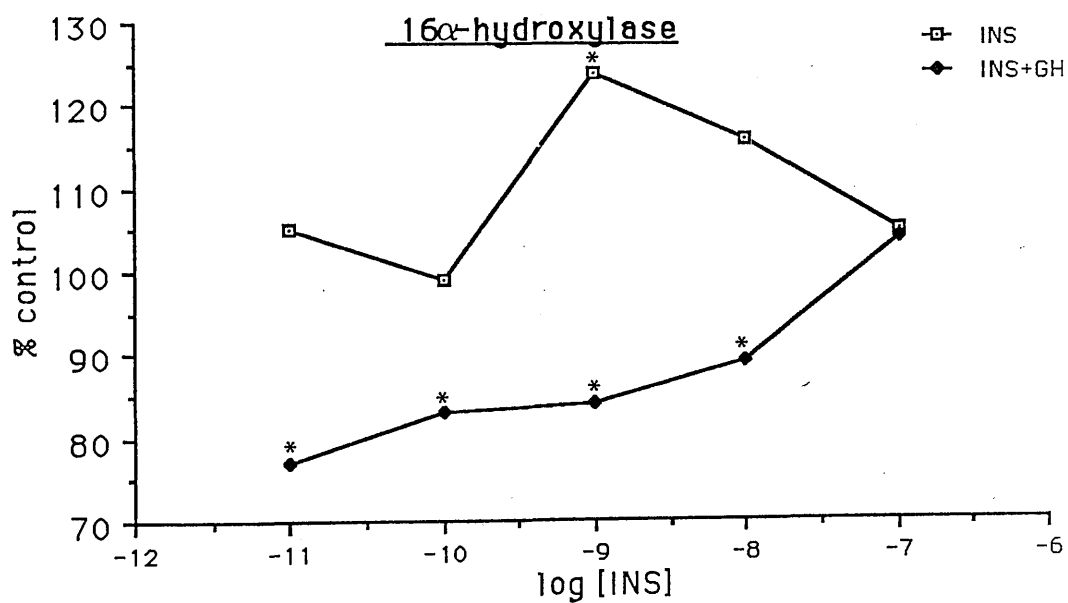
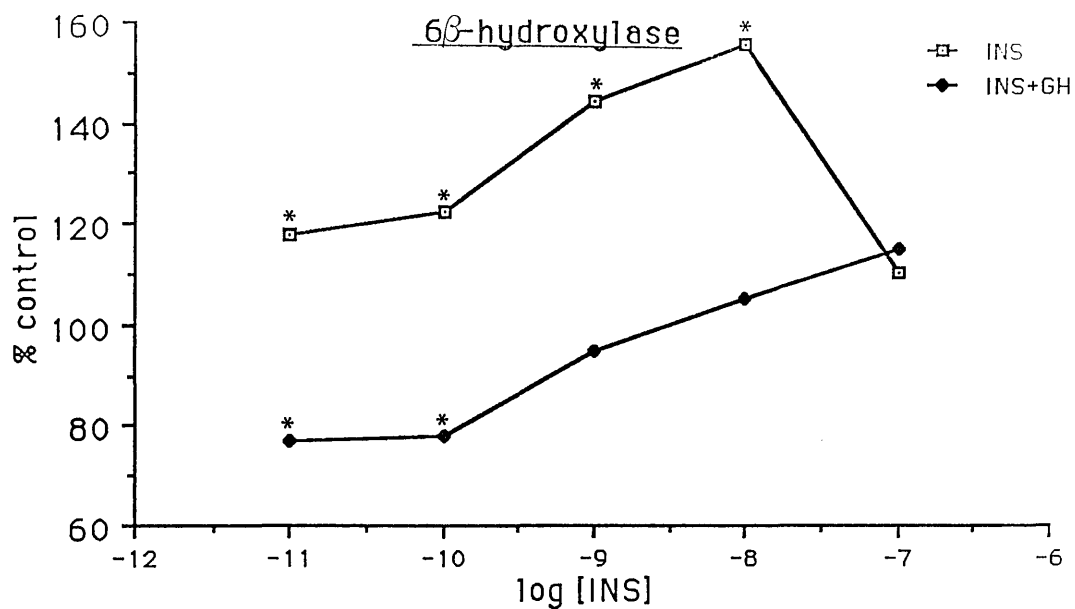


TABLE 11 : Dose-dependent effects of 30 minute preincubation with A) 10^{-11} M- 10^{-7} M INSULIN and B) 10^{-11} M- 10^{-7} M INSULIN + 10^{-9} M GROWTH HORMONE on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

A) Enzyme activities (pmoles/minute/million cells)					
TREATMENT	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
Control (A)	45 \pm 2	52 \pm 1	47 \pm 3	84 \pm 2	112 \pm 4
10^{-11} M (B)	46 \pm 1	61 \pm 4*	49 \pm 3	76 \pm 1*	127 \pm 1*
10^{-10} M (C)	46 \pm 2	64 \pm 4*	46 \pm 5	86 \pm 2	120 \pm 1*
10^{-9} M (D)	53 \pm 5*	75 \pm 4*	58 \pm 4*	100 \pm 1*	137 \pm 1*
10^{-8} M (E)	61 \pm 1*	82 \pm 5*	54 \pm 6	107 \pm 13*	134 \pm 3*
10^{-7} M (F)	51 \pm 3*	58 \pm 1	49 \pm 1	71 \pm 9*	130 \pm 1*

B) Enzyme activities (pmoles/minute/million cells)					
TREATMENT	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
Control (A)	44 \pm 2	38 \pm 2	36 \pm 1	50 \pm 2	103 \pm 2
10^{-11} M+GH (B)	45 \pm 3	30 \pm 1*	28 \pm 3*	30 \pm 1*	95 \pm 3
10^{-10} M+GH (C)	46 \pm 2	31 \pm 2*	30 \pm 2*	28 \pm 3*	95 \pm 9
10^{-9} M +GH (D)	45 \pm 1	36 \pm 1	31 \pm 2*	29 \pm 1*	105 \pm 2
10^{-8} M +GH (E)	45 \pm 2	41 \pm 2	32 \pm 3*	29 \pm 4*	90 \pm 8*
10^{-7} M +GH (F)	45 \pm 4	43 \pm 4	36 \pm 4	46 \pm 7	108 \pm 6

Results are expressed as mean \pm s.d. (N=3); * = P < 0.05 of relevant control values.

Duncan's Multiple Range Test :

A)

7 α -OHase	<u>A B C F D E</u>
6 β -OHase	<u>A F B C D E</u>
16 α -OHase	<u>C A B F D E</u>
17-OHSD	<u>F B A C D E</u>
5 α -red	<u>A C B F E D</u>

B)

7 α -OHase	<u>A B D E F C</u>
6 β -OHase	<u>B C D A E F</u>
16 α -OHase	<u>B C D E A F</u>
17-OHSD	<u>C D E B F A</u>
5 α -red	<u>E B C A D F</u>

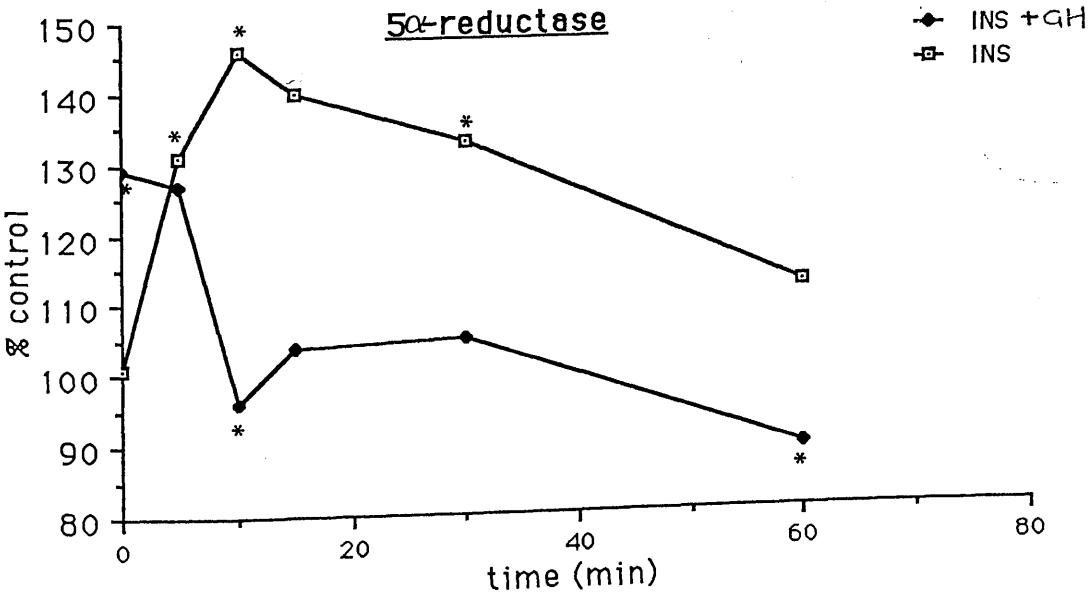
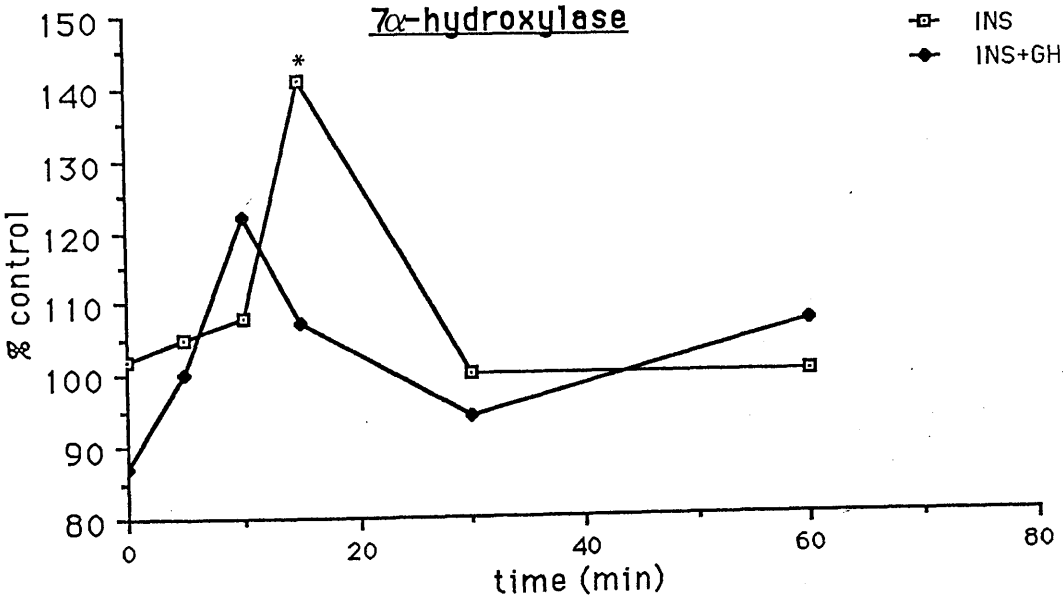
antagonising the stimulatory action of insulin, these effects were non-sex-specific in nature since both male- and female-specific activities were affected in an identical manner.

3.2.2 TIME COURSE OF THE INTERACTION BETWEEN 10^{-11} M INSULIN AND 10^{-9} M GROWTH HORMONE

Hepatocytes were pretreated with 10^{-11} M insulin with or without 10^{-9} M growth hormone over a period of 0-60 minutes. The effects of incubating hepatocytes with 10^{-11} M insulin alone demonstrated maximal stimulation at 15 minutes of incubation (Table 12A), and enzyme activity remained elevated until 30 minutes of incubation for 5 α -reductase (Figure 14B), 6 β -hydroxylase (Figure 14C) and 16 α -hydroxylase (Figure 14D) activities. All enzyme activities had returned to basal levels by 60 minutes of incubation (Figure 14).

Pretreatment of hepatocytes with a combination of 10^{-11} M insulin and 10^{-9} M growth hormone resulted in an antagonism of the stimulatory effects of insulin (Table 12B). This antagonistic action of growth hormone was evident from as early as 5 minutes of incubation, as is the case with 16 α -hydroxylase and 17-oxosteroid oxidoreductase activities (Figures 14D and 14E respectively), and from 10 minutes with 7 α -hydroxylase, 5 α -reductase and 6 β -hydroxylase activities (Figures 14A, 14B and 14C respectively).

FIGURE 14 : Time course of the effects of preincubation with 10^{-11} M INSULIN in the absence and presence of 10^{-9} M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 12



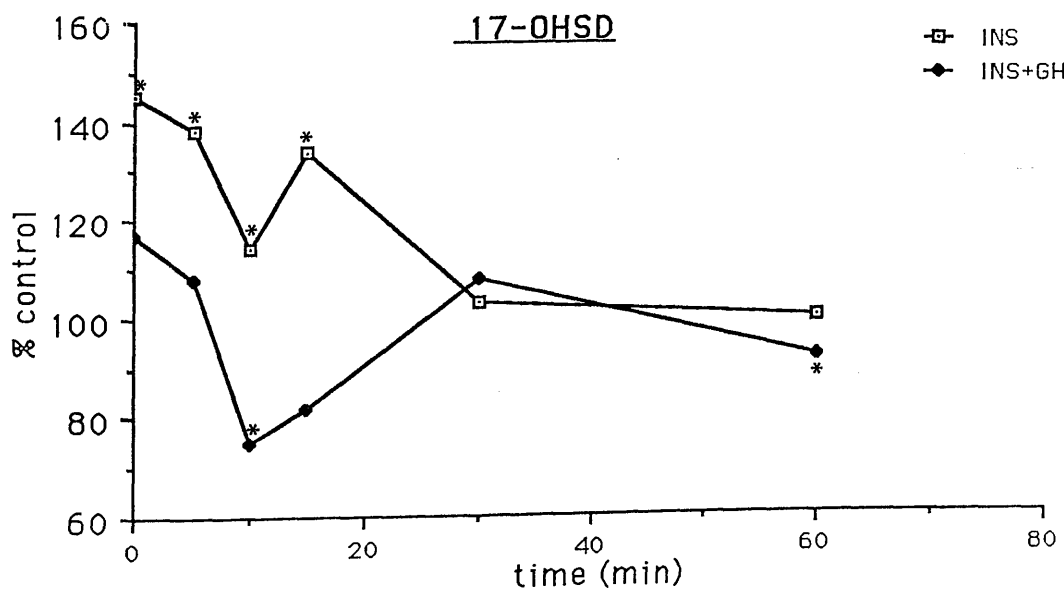
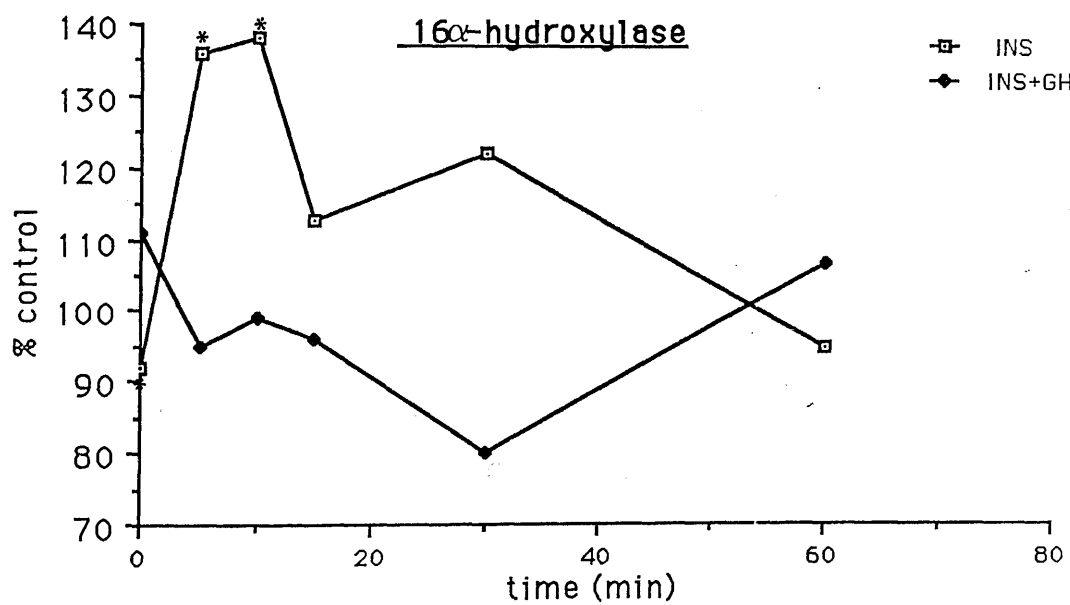
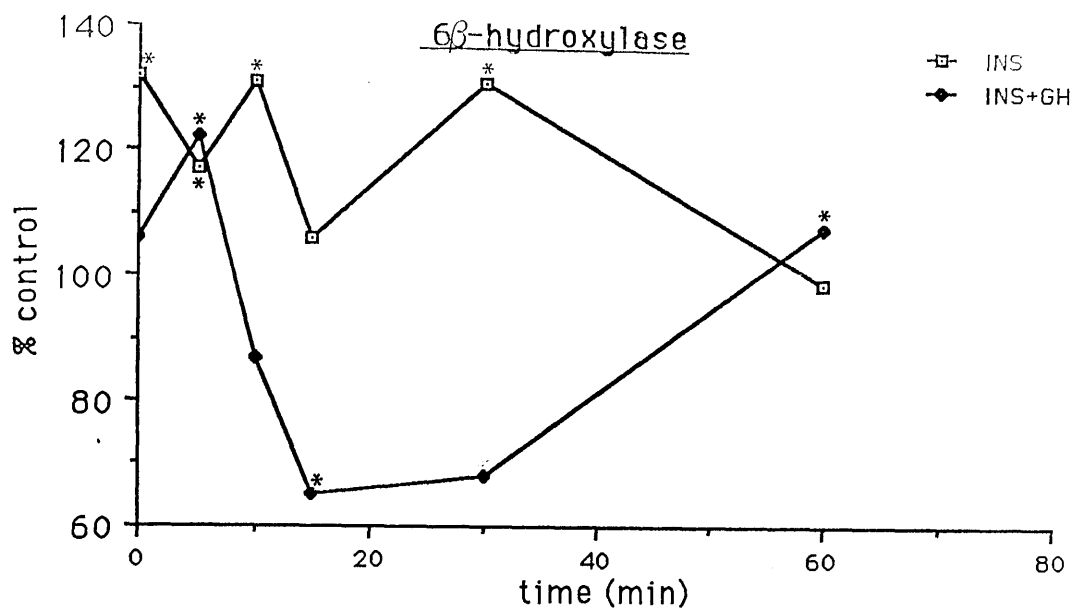


TABLE 12 : Effects of preincubation with A) 10^{-11} M INSULIN and B) 10^{-11} M INSULIN + 10^{-9} M GH over 0 to 60 minutes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

A)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
0C		57 \pm 5	62 \pm 4	51 \pm 3	78 \pm 4	107 \pm 3
0INS		58 \pm 4	82 \pm 9*	47 \pm 1*	113 \pm 5*	109 \pm 11
5C		62 \pm 7	71 \pm 10	47 \pm 6	87 \pm 9	90 \pm 16
5INS		65 \pm 2	83 \pm 2*	64 \pm 7*	120 \pm 4*	118 \pm 16*
10C		51 \pm 3	81 \pm 6	50 \pm 4	107 \pm 2	91 \pm 8
10INS		55 \pm 6	106 \pm 1*	69 \pm 1*	122 \pm 2*	133 \pm 7*
15C		61 \pm 9	64 \pm 6	55 \pm 8	96 \pm 1	115 \pm 22
15INS		86 \pm 7*	68 \pm 5	62 \pm 4	129 \pm 12*	139 \pm 12
30C		69 \pm 7	61 \pm 6	46 \pm 3	115 \pm 6	107 \pm 16
30INS		70 \pm 4	80 \pm 12*	56 \pm 7	118 \pm 5	143 \pm 12*
60C		57 \pm 9	76 \pm 4	43 \pm 2	111 \pm 6	107 \pm 12
60INS		57 \pm 6	75 \pm 7	41 \pm 2	112 \pm 4	120 \pm 15

B)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
0C		49 \pm 8	42 \pm 4	26 \pm 6	95 \pm 16	98 \pm 12
0INS+GH		43 \pm 3	44 \pm 4	29 \pm 2	111 \pm 1	127 \pm 3*
5C		40 \pm 4	41 \pm 5	38 \pm 4	119 \pm 11	120 \pm 7
5INS+GH		40 \pm 2	50 \pm 6*	36 \pm 4	129 \pm 13	152 \pm 5*
10C		46 \pm 8	56 \pm 7	34 \pm 3	132 \pm 4	125 \pm 1
10INS+GH		56 \pm 4	48 \pm 2	34 \pm 2	99 \pm 21*	121 \pm 6
15C		35 \pm 4	67 \pm 2	34 \pm 1	126 \pm 18	117 \pm 15
15INS+GH		38 \pm 5	43 \pm 7*	33 \pm 4	104 \pm 18	122 \pm 5
30C		45 \pm 3	70 \pm 6	41 \pm 1	84 \pm 14	108 \pm 1
30INS+GH		45 \pm 4	70 \pm 8	41 \pm 1	91 \pm 6	114 \pm 7
60C		41 \pm 1	52 \pm 1	37 \pm 3	119 \pm 2	96 \pm 10
60INS+GH		41 \pm 3	56 \pm 3*	40 \pm 4	109 \pm 8*	83 \pm 4*

Results are expressed as mean \pm s.d. (N=3); *= $P < 0.05$ as compared to relevant control values (C=control).

3.2.3 TIME COURSE OF THE INTERACTION BETWEEN 10^{-9} M INSULIN AND 10^{-9} M GROWTH HORMONE

Hepatocytes were pretreated with 10^{-9} M insulin in the absence or presence of 10^{-9} M growth hormone over a period of 0 to 60 minutes.

Incubation with 10^{-9} M insulin (Table 13A) resulted in significant increases in all enzyme activities, as is illustrated in Figure 15, and this activity peaked after 30 minutes of incubation, moving towards basal levels thereafter.

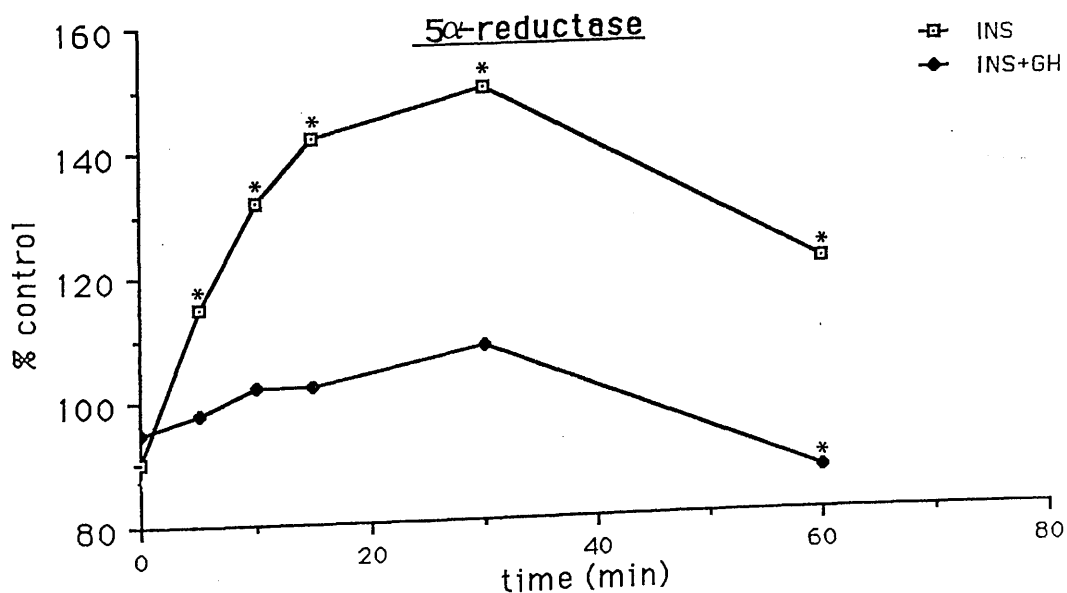
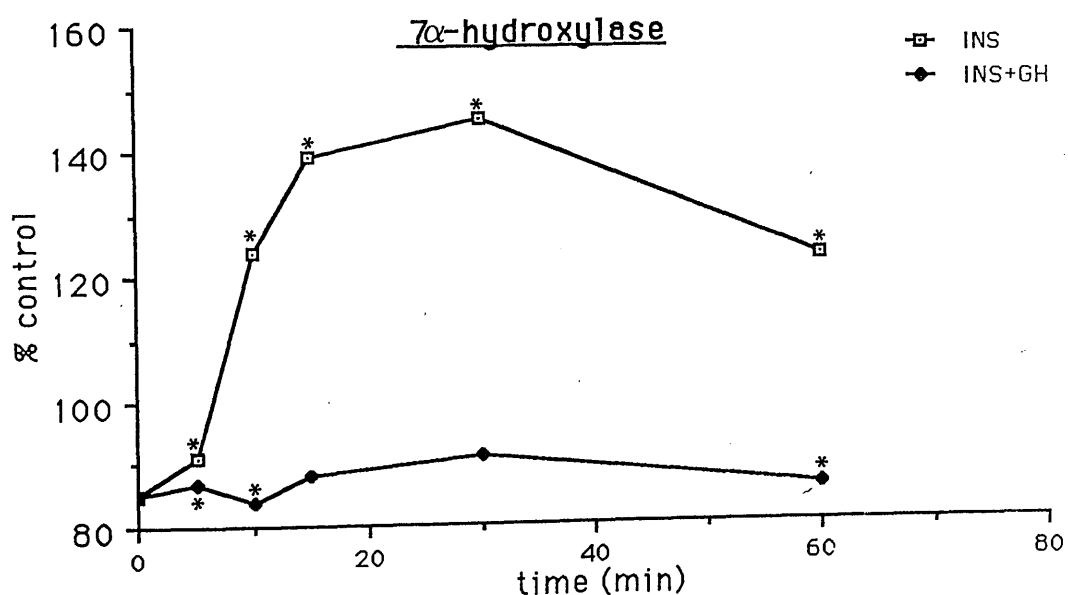
Preincubation with a combination of insulin and growth hormone (Table 13B) showed marked decreases in enzyme activity, as is shown in Figure 15. The effect of growth hormone in antagonising the stimulatory action of insulin was apparent early in the time course, and was maintained throughout the 60 minutes of incubation, except with 17-oxosteroid oxidoreductase activity where this effect was lost after 60 minutes of incubation (Figure 15E).

3.2.4 TIME COURSE OF THE INTERACTION BETWEEN 10^{-7} M INSULIN AND 10^{-9} M GROWTH HORMONE

Table 14A shows the effects of pretreatment of hepatocytes with 10^{-7} M insulin with or without the presence of 10^{-9} M growth hormone for 0-60 minutes. Incubation with 10^{-7} M insulin exhibited significant increases in enzyme activity in all cases (Figure 16), showing maxima at 30 minutes of incubation, except for 6β -hydroxylase activity

FIGURE 15 : Time course of the effects of preincubation with 10^{-9} M INSULIN in the absence and presence of 10^{-9} M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 13



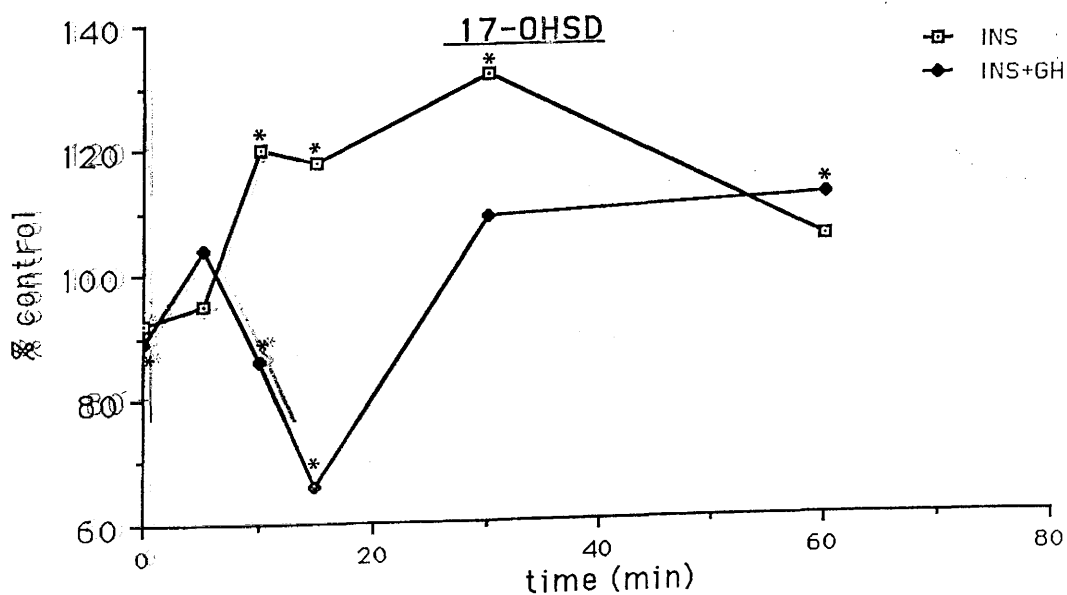
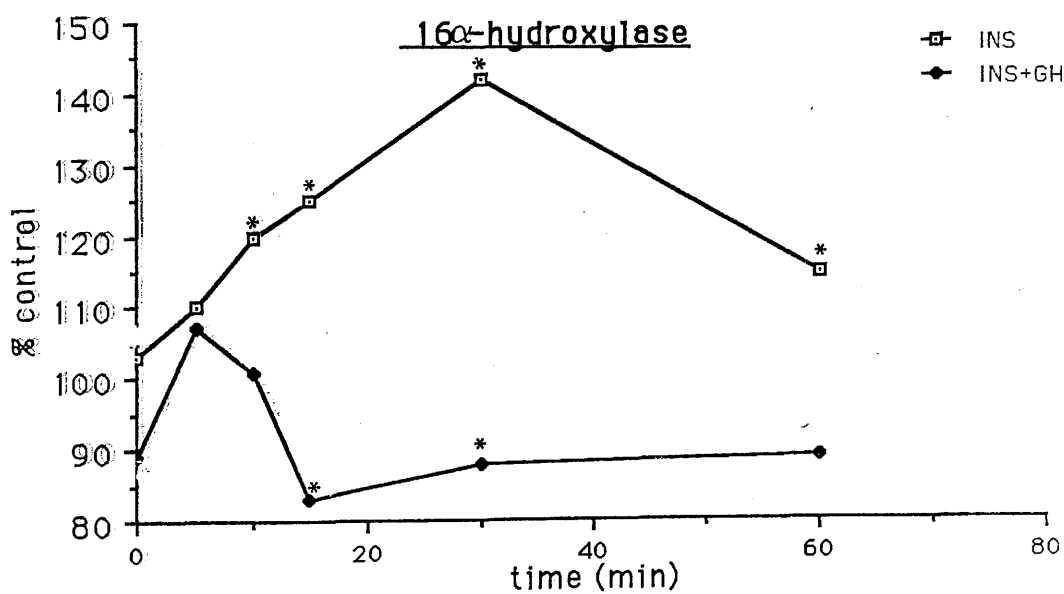
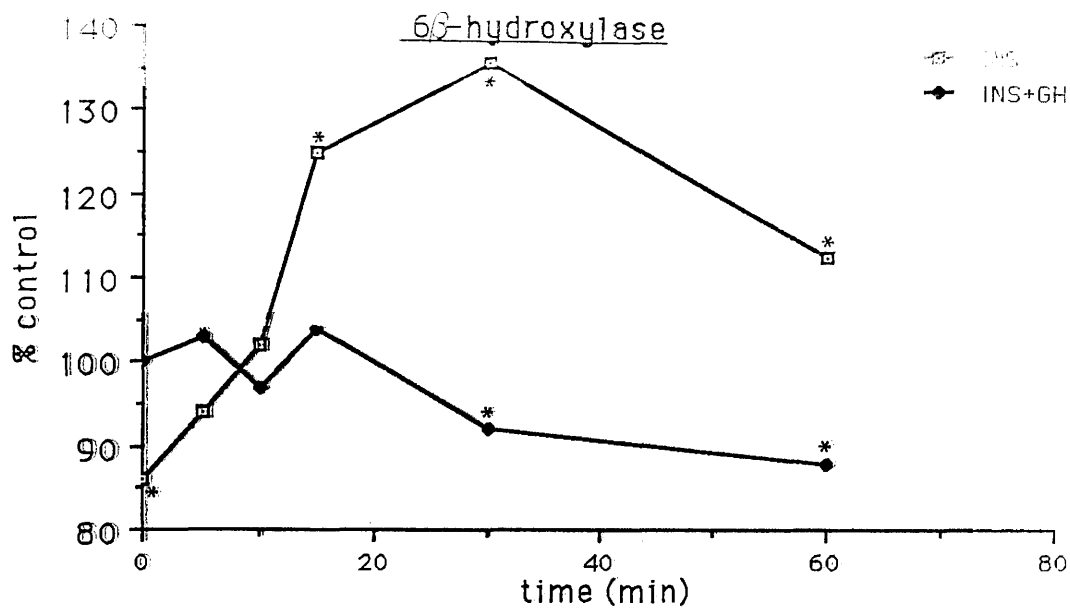
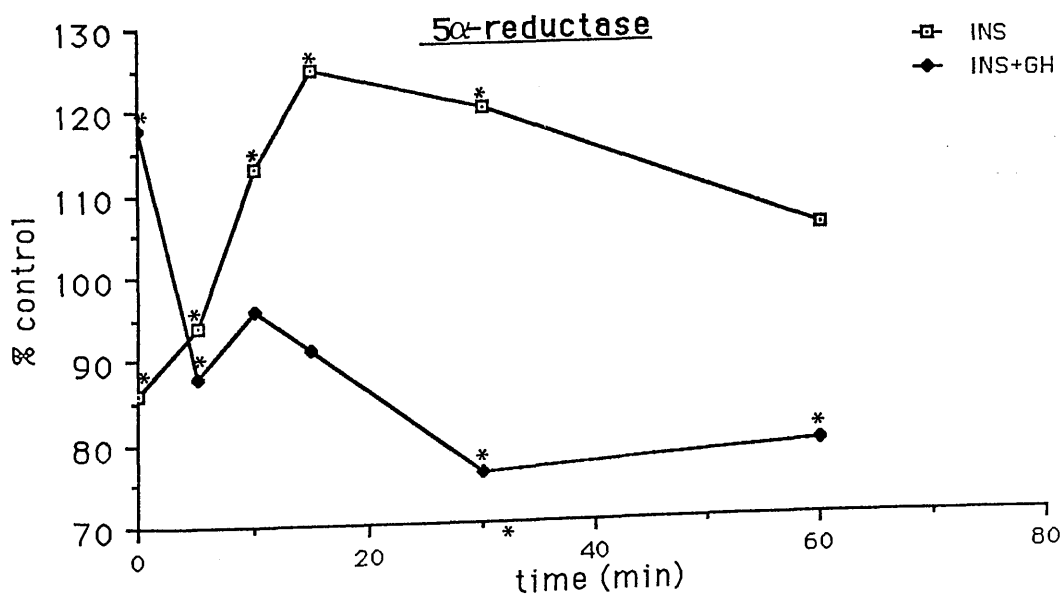
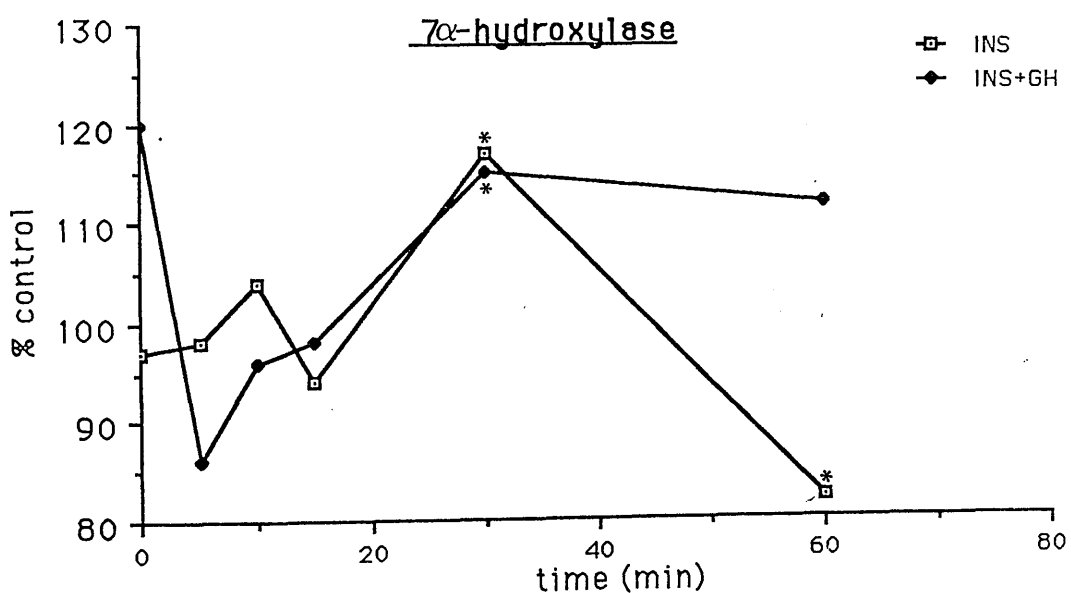


TABLE 13 : Effects of preincubation with A) 10^{-9} M INSULIN and B) 10^{-9} M INSULIN + 10^{-9} M GROWTH HORMONE over 0-60 minutes on 7α -, 6β - and 16α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5α -reductase activities.

A)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7α -OHase	6β -OHase	16α -OHase	17-OHSD	5α -red
0C		38 \pm 4	66 \pm 5	36 \pm 1	52 \pm 5	91 \pm 12
0INS		32 \pm 6	57 \pm 1*	37 \pm 2	48 \pm 5	82 \pm 10
5C		32 \pm 1	60 \pm 3	40 \pm 5	63 \pm 1	79 \pm 4
5INS		29 \pm 1*	56 \pm 4	44 \pm 1	60 \pm 3	91 \pm 1*
10C		28 \pm 2	57 \pm 2	48 \pm 1	46 \pm 2	83 \pm 4
10INS		35 \pm 4*	58 \pm 1	58 \pm 6*	56 \pm 4*	110 \pm 4*
15C		36 \pm 1	67 \pm 1	45 \pm 6	53 \pm 4	82 \pm 2
15INS		50 \pm 1*	44 \pm 4*	56 \pm 1*	63 \pm 1*	116 \pm 3*
30C		28 \pm 1	59 \pm 5	43 \pm 2	59 \pm 2	72 \pm 5
30INS		41 \pm 2*	80 \pm 1*	61 \pm 2*	78 \pm 7*	108 \pm 1*
60C		29 \pm 2	54 \pm 5	57 \pm 2	62 \pm 2	76 \pm 12
60INS		36 \pm 2*	61 \pm 3*	66 \pm 4*	65 \pm 4	92 \pm 5*
B)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7α -OHase	6β -OHase	16α -OHase	17-OHSD	5α -red
0C		36 \pm 5	49 \pm 2	47 \pm 4	62 \pm 3	82 \pm 11
0INS+GH		31 \pm 3	49 \pm 3	42 \pm 2	56 \pm 1*	78 \pm 7
5C		30 \pm 1	54 \pm 5	40 \pm 1	53 \pm 1	83 \pm 10
5INS+GH		26 \pm 1*	56 \pm 1	43 \pm 2	56 \pm 4	82 \pm 7
10C		43 \pm 3	60 \pm 2	50 \pm 2	64 \pm 4	92 \pm 8
10INS+GH		36 \pm 4*	58 \pm 2	51 \pm 1	55 \pm 6*	94 \pm 4
15C		39 \pm 8	60 \pm 4	36 \pm 2	58 \pm 3	101 \pm 4
15INS+GH		34 \pm 1	62 \pm 6	30 \pm 2*	38 \pm 1*	103 \pm 5
30C		40 \pm 3	72 \pm 5	50 \pm 4	48 \pm 2	94 \pm 2
30INS+GH		36 \pm 4	55 \pm 5*	44 \pm 1*	53 \pm 7	102 \pm 10
60C		42 \pm 1	62 \pm 1	53 \pm 6	55 \pm 2	103 \pm 5
60INS+GH		36 \pm 2*	55 \pm 4*	47 \pm 1	61 \pm 4*	90 \pm 1*

Results are expressed as mean \pm s.d. (N=3); *= $P < 0.05$ as compared to relevant control values (C=control).

FIGURE 16 : Time course of the effects of preincubation with 10^{-7} M INSULIN in the absence and presence of 10^{-9} M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 14



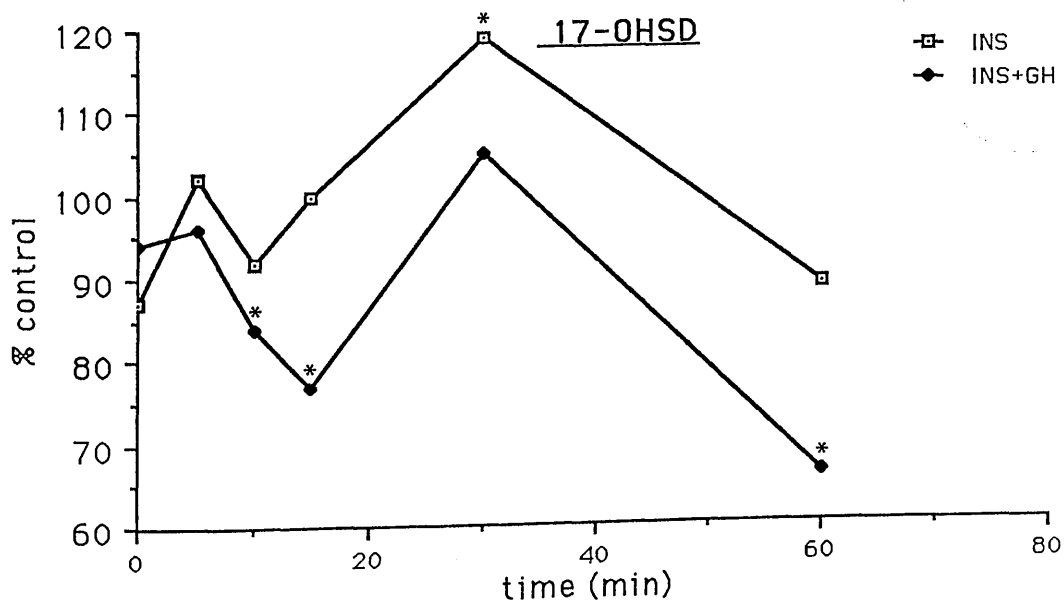
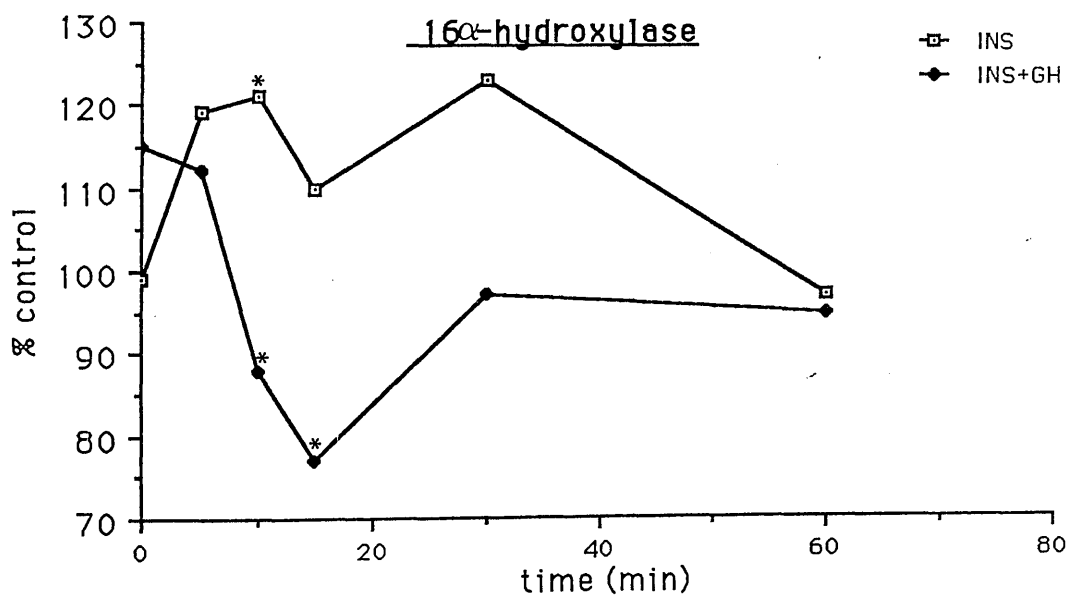
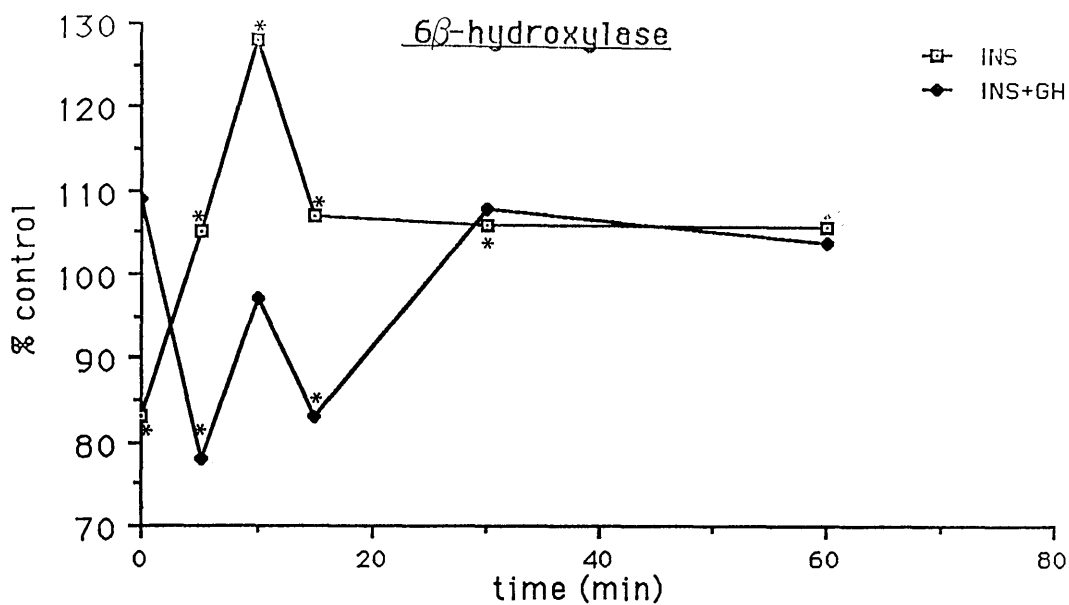


TABLE 14 : Effects of preincubation with A) 10^{-7} M INSULIN and B) 10^{-7} M INSULIN + 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 7α -, 6β -, 16α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5α -reductase activities.

A)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7α -OHase	6β -OHase	16α -OHase	17-OHSD	5α -red
0C		78±6	64±1	55±10	54±9	120±1
0INS		75±3	54±5*	55±4	48±6	103±2*
5C		80±4	76±2	44±4	40±1	119±3
5INS		79±4	80±2*	52±6	40±4	111±6*
10C		78±4	56±3	52±6	47±4	122±8
10INS		80±4	72±5*	64±2*	43±3	137±5*
15C		78±8	48±1	48±2	44±2	113±8
15INS		73±6	52±2*	54±8	45±5	142±15*
30C		59±1	59±1	46±6	42±2	119±9
30INS		69±7*	63±2*	58±8	50±3*	139±12*
60C		88±4	62±2	38±1	72±9	116±16
60INS		73±2*	62±7	37±4	64±10	125±3

B)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7α -OHase	6β -OHase	16α -OHase	17-OHSD	5α -red
0C		70±6	52±13	50±10	46±7	93±5
0INS+GH		84±3	56±4	57±5	44±5	110±5*
5C		88±9	65±3	46±10	52±6	103±6
5INS+GH		76±6	50±7*	52±3	50±6	90±7*
10C		78±3	62±6	68±3	46±3	98±2
10INS+GH		75±6	60±4	60±5*	39±5*	95±9
15C		72±5	66±7	53±2	46±3	98±10
15INS+GH		71±3	55±5*	41±4*	36±3*	90±5
30C		69±3	62±8	50±5	42±4	110±13
30INS+GH		80±7*	67±4	48±6	44±5	84±7*
60C		74±9	56±3	63±4	62±6	82±3
60INS+GH		82±5	58±7	60±4	41±5*	65±6*

Results are expressed as mean ± s.d. (N=3); *= $P < 0.05$ as compared to relevant control values (C=control).

(Figure 16C) where activity peaked after 10 minutes of incubation. The stimulatory effect of insulin was lost by 60 minutes of incubation in all cases.

Growth hormone antagonised the stimulatory effect of 10^{-7} M insulin on all enzyme activities (Table 14B), and this inhibition was apparent at the earlier incubation times, as is illustrated in Figure 16. No significant effect was however observed with 7 α -hydroxylase activity (Figure 16A).

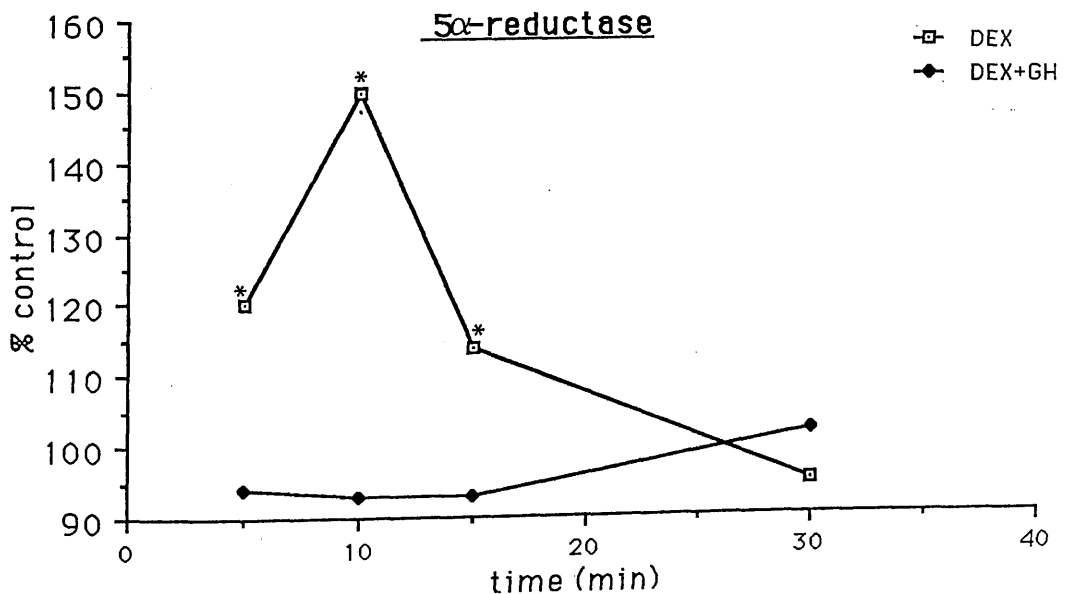
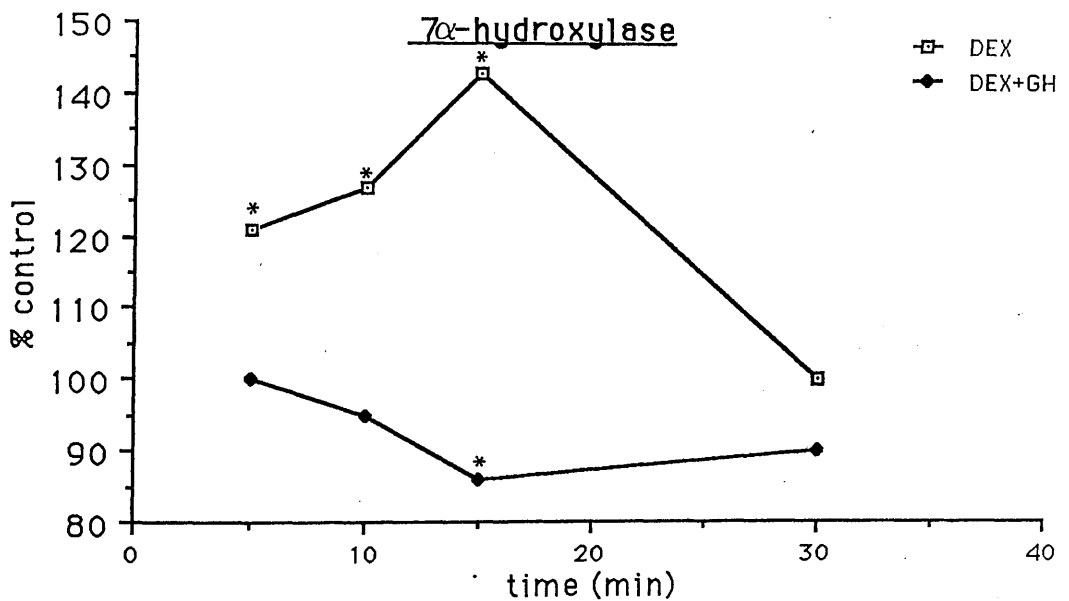
3.3 DEXAMETHASONE

3.3.1 TIME COURSE OF THE EFFECTS OF 10^{-8} M DEXAMETHASONE AND ITS INTERACTION WITH GROWTH HORMONE

Preincubation of hepatocytes with the synthetic glucocorticoid dexamethasone at a concentration of 10^{-8} M over 0-30 minutes caused marked increases in enzyme activity (Table 15). This stimulation of enzyme activity was non-specific in nature as both male- and female-specific activities were affected in the same way, as is illustrated in Figure 17. This stimulation of enzyme activity was optimal early in the time course, returning to basal levels by 30 minutes in most cases (Figure 17), except for 16 α -hydroxylase activity (Figure 17D) where the stimulatory effect was maintained.

Inclusion of 10^{-9} M growth hormone in the incubation medium in combination with 10^{-8} M dexamethasone resulted in a marked reduction in enzyme activity with respect to the response obtained on incubation with dexamethasone alone

FIGURE 17 : Effects of preincubation with 10^{-8} M DEXAMETHASONE in the absence and presence of 10^{-9} M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 15



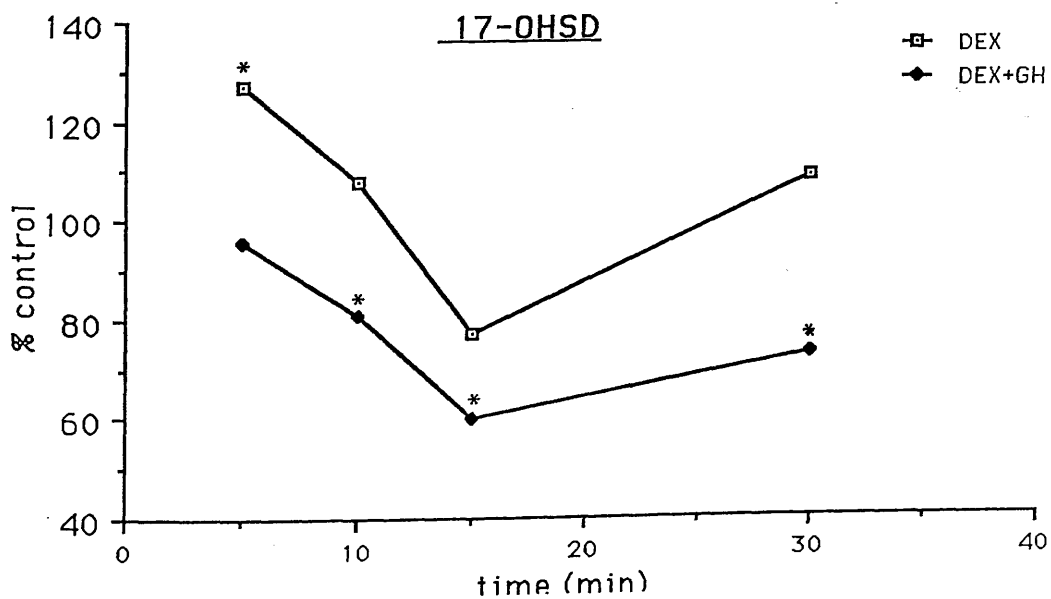
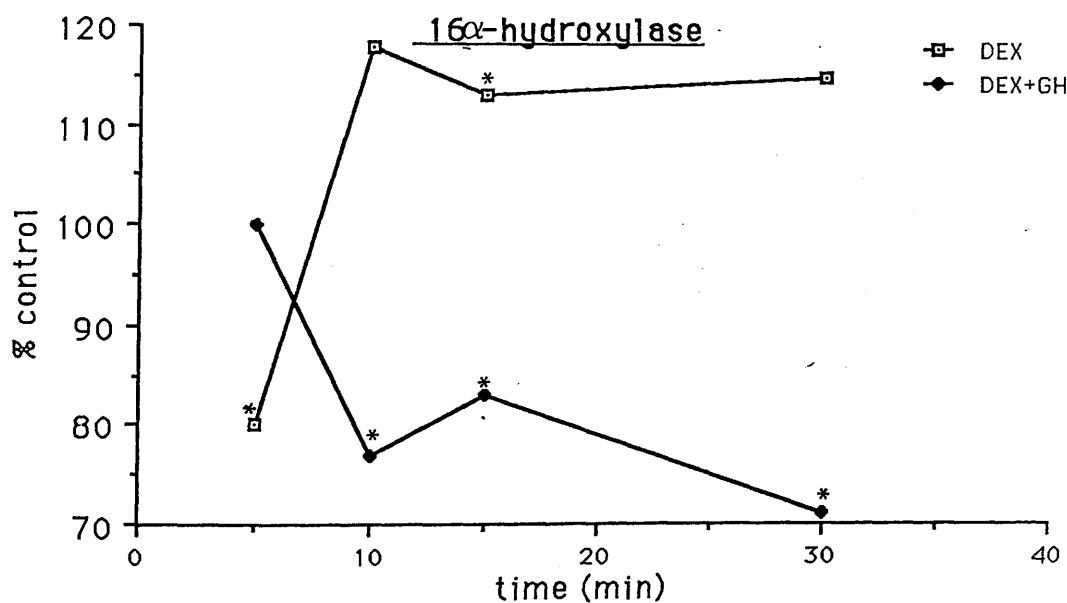
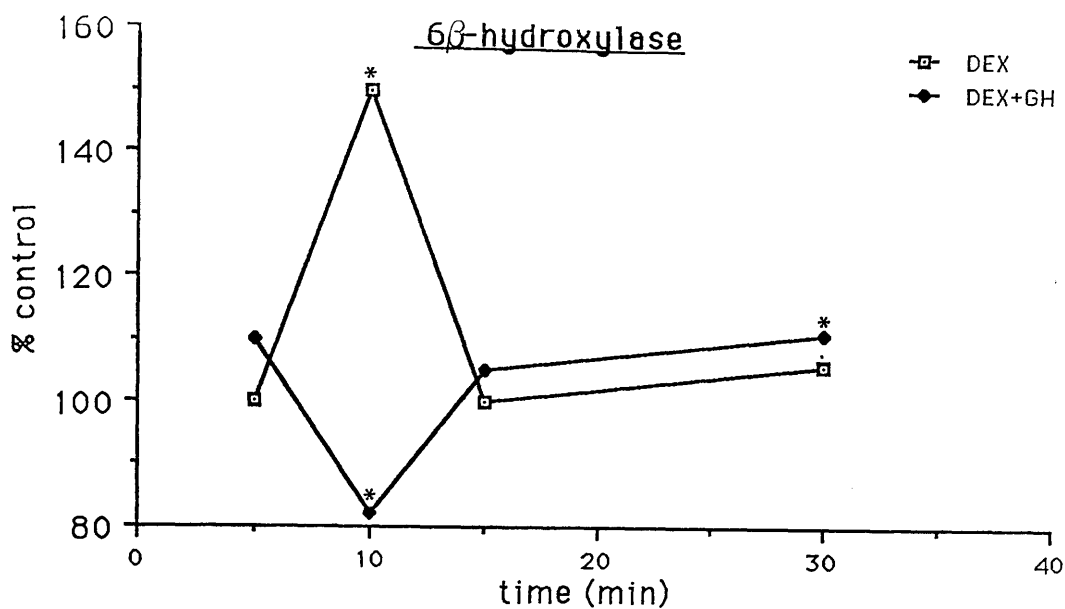


TABLE 15 : Effects of preincubation with 10^{-8} M DEXAMETHASONE +/- 10^{-9} M GH over 5 to 30 minutes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

Enzyme activities (pmoles/minute/million cells)					
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
5C	56 \pm 2	68 \pm 16	60 \pm 6	88 \pm 8	102 \pm 5
5DEX	68 \pm 8*	68 \pm 14	48 \pm 8*	112 \pm 4*	122 \pm 15*
10C	60 \pm 7	64 \pm 8	68 \pm 10	96 \pm 7	120 \pm 18
10DEX	76 \pm 6*	96 \pm 2*	80 \pm 2	104 \pm 6	180 \pm 19*
15C	56 \pm 10	72 \pm 8	60 \pm 2	104 \pm 20	100 \pm 6
15DEX	80 \pm 2*	72 \pm 10	72 \pm 2*	80 \pm 2	114 \pm 7*
30C	76 \pm 1	68 \pm 10	52 \pm 8	92 \pm 10	112 \pm 12
30DEX	76 \pm 4	72 \pm 8	60 \pm 4	100 \pm 4	106 \pm 15
5C	76 \pm 7	76 \pm 8	68 \pm 7	112 \pm 20	146 \pm 14
5DEX+GH	76 \pm 8	84 \pm 6	68 \pm 12	108 \pm 6	138 \pm 4
10C	84 \pm 12	88 \pm 2	88 \pm 10	124 \pm 8	134 \pm 12
10DEX+GH	80 \pm 12	72 \pm 6*	68 \pm 2*	100 \pm 8*	144 \pm 6
15C	84 \pm 5	76 \pm 14	72 \pm 10	120 \pm 3	142 \pm 22
15DEX+GH	72 \pm 8*	80 \pm 14	60 \pm 2*	72 \pm 2*	132 \pm 20
30C	80 \pm 20	72 \pm 2	68 \pm 8	120 \pm 1	118 \pm 6
30DEX+GH	72 \pm 14	80 \pm 2*	48 \pm 2*	88 \pm 2*	120 \pm 22

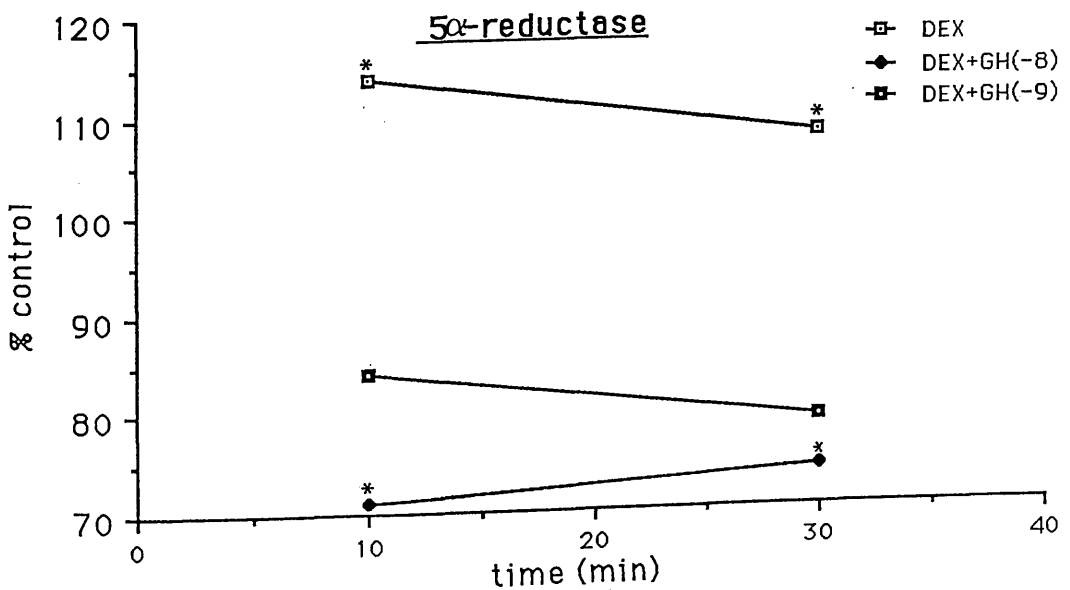
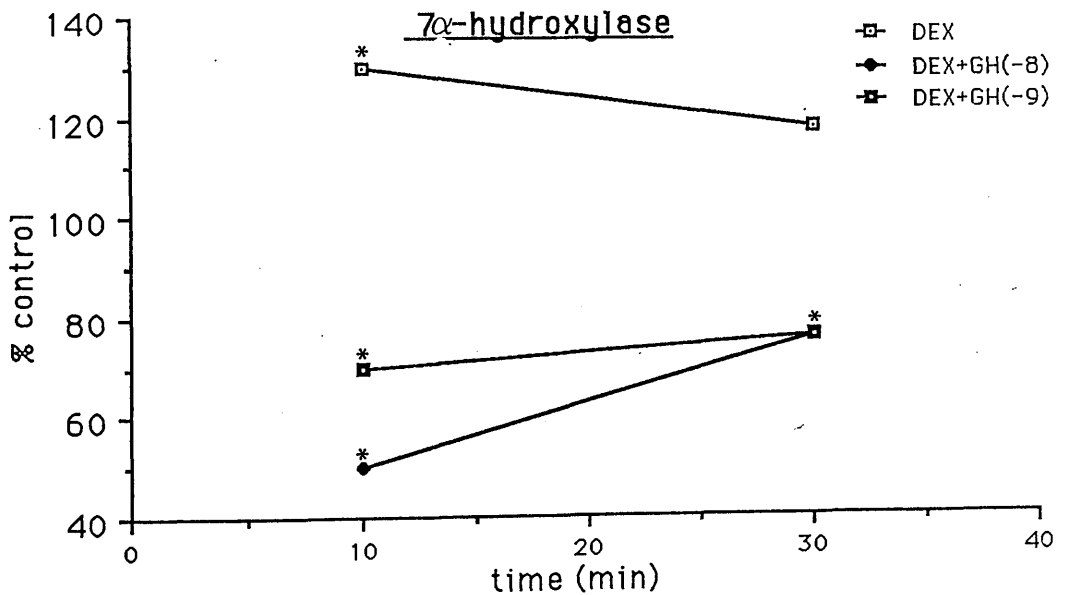
Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values (C=control).

(Table 15), as shown in Figure 17. Growth hormone appears to modify the stimulatory action of dexamethasone non-specifically since both male- and female-specific activities are affected in a similar manner.

3.3.2 A COMPARISON OF THE INTERACTION BETWEEN 10^{-8} M DEXAMETHASONE AND DIFFERING CONCENTRATIONS OF GROWTH HORMONE

Table 16 shows the effects of incubation of hepatocytes with 10^{-8} M dexamethasone, and the interaction observed between growth hormone at 10^{-9} M and 10^{-8} M. As illustrated in Figure 18, incubation with dexamethasone stimulated all enzyme activities significantly. Co-incubation with 10^{-9} M growth hormone significantly reduced the stimulatory effects of dexamethasone in all cases (Figure 18) by up to 70%, as is the case with 6 β -hydroxylase activity (Figure 18C). Incubation of hepatocytes with a combination of 10^{-8} M dexamethasone and 10^{-8} M growth hormone resulted in an enhancement of the interaction obtained with 10^{-9} M growth hormone in that enzyme activities were further decreased by approximately 10-20% (Figure 18). Thus growth hormone appeared to display a certain degree of dose-dependency in its effect on the stimulatory response to dexamethasone.

FIGURE 18 : A comparison of the effects of preincubation with 10^{-8} M DEXAMETHASONE in the absence and presence of 10^{-9} M or 10^{-8} M GROWTH HORMONE for 10 and 30 minutes on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 16



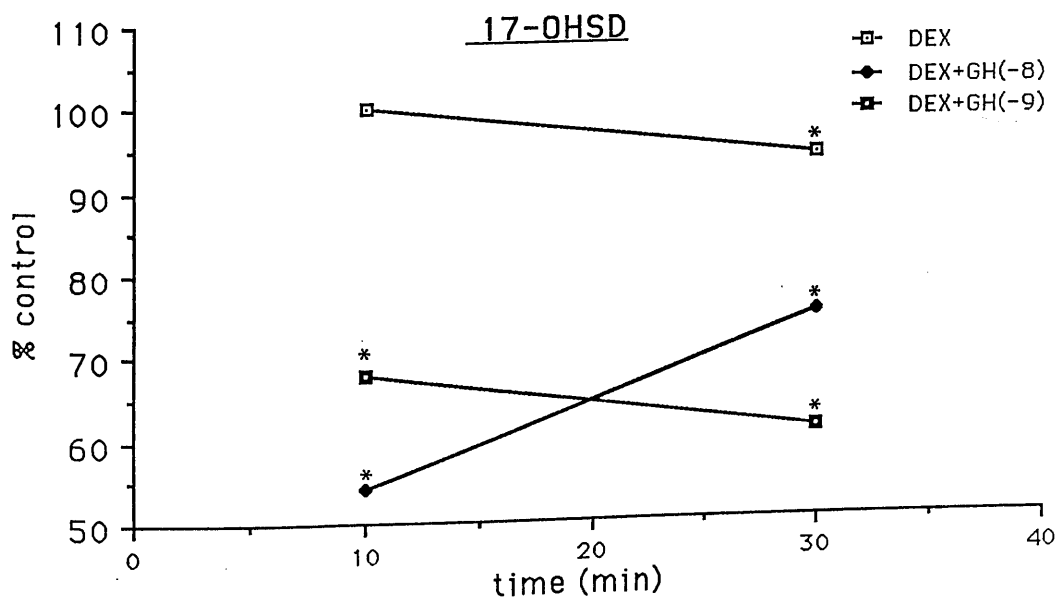
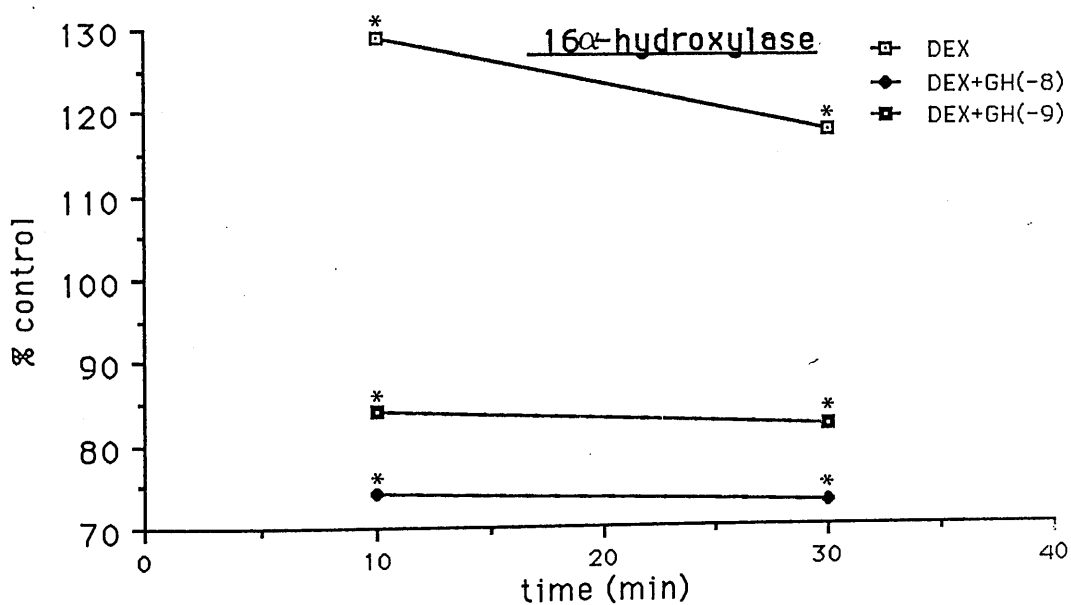
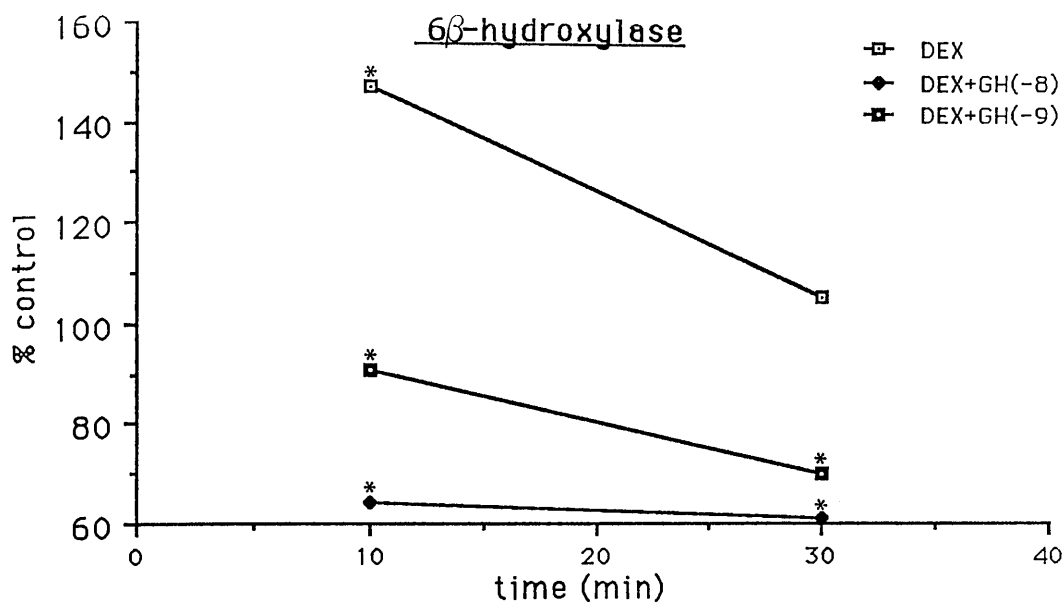


TABLE 16 : Effects of preincubation with 10^{-8} M DEXAMETHASONE in the absence or presence of 10^{-8} M or 10^{-9} M GROWTH HORMONE for 10 and 30 minutes on 7α -, 6β -, 16α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5α -reductase activities.

TREATMENT	Enzyme activities (pmoles/minute/million cells)				
	7α -OHase	6β -OHase	16α -OHase	17-OHSD	5α -red
10 C	30 \pm 1	45 \pm 7	42 \pm 8	45 \pm 4	101 \pm 2
10 DEX	39 \pm 3*	66 \pm 10*	54 \pm 4*	45 \pm 4	115 \pm 8*
10 C	60 \pm 15	66 \pm 2	57 \pm 4	66 \pm 6	116 \pm 4
10 DEX, -8GH	30 \pm 2*	42 \pm 2*	42 \pm 2*	36 \pm 3*	82 \pm 2*
10 DEX, -9GH	42 \pm 1*	60 \pm 4*	48 \pm 2*	45 \pm 6*	98 \pm 1
30 C	33 \pm 1	51 \pm 4	51 \pm 2	51 \pm 2	89 \pm 1
30 DEX	39 \pm 4	54 \pm 5	60 \pm 1*	48 \pm 2*	96 \pm 2*
30 C	51 \pm 4	69 \pm 2	66 \pm 2	84 \pm 2	153 \pm 25
30 DEX, -8GH	39 \pm 4*	42 \pm 4*	48 \pm 2*	63 \pm 4*	113 \pm 10*
30 DEX, -9GH	48 \pm 6	48 \pm 2*	54 \pm 2*	51 \pm 1*	121 \pm 4

Results are expressed as mean \pm s.d. (N=3); *= $P < 0.05$ as compared to relevant control values (C=control).

3.3.3 PRETREATMENT OF HEPATOCYTES WITH DEXAMETHASONE
FOR 24,48 AND 72h

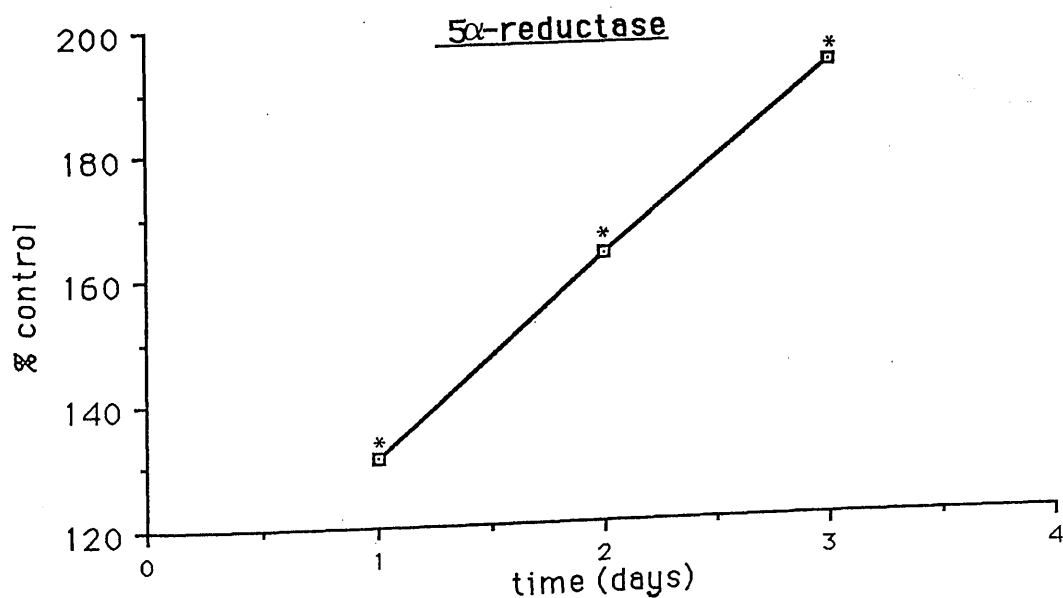
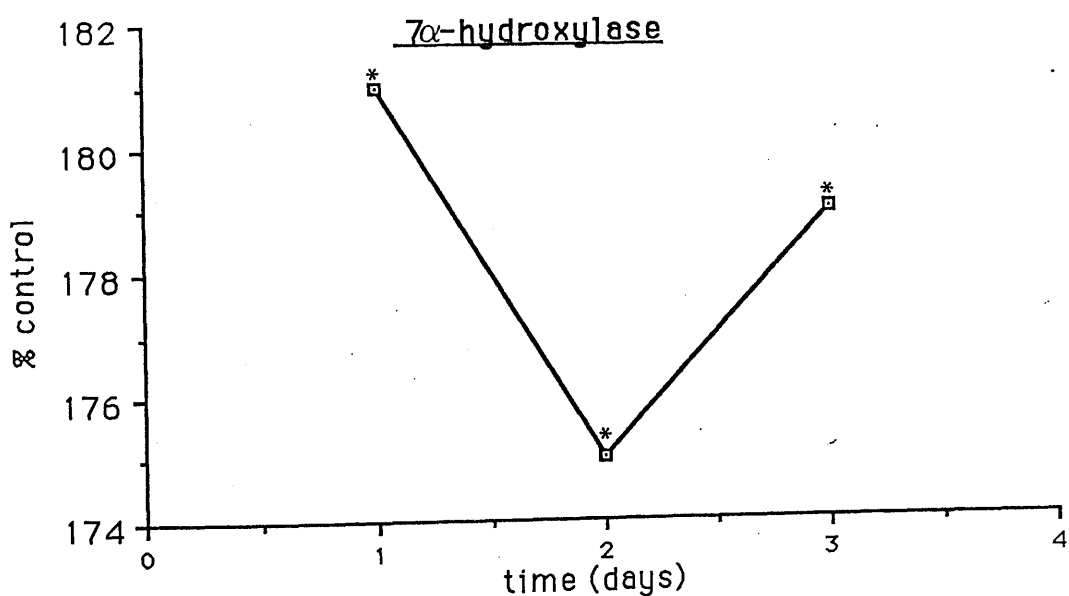
Hepatocytes were pretreated with dexamethasone for 24,48 and 72h as described in Section 2.5.2. Table 17 shows that incubation with dexamethasone markedly stimulated enzyme activity. 5 α -reductase, 6 β -hydroxylase and 17-oxosteroid oxidoreductase (Figures 19B, 19C and 19E respectively) showed a gradual increase in activity with time, whilst 7 α -hydroxylase (Figure 19A) and 16 α -hydroxylase (Figure 19D) reached a peak at 24h of incubation that was maintained throughout the incubation period. Thus dexamethasone appears to be a potent inducer of steroid metabolism in cultured hepatocytes.

3.3.4 PRETREATMENT OF HEPATOCYTES WITH 10⁻⁸M
DEXAMETHASONE FOR 24,48 AND 72h FOLLOWED BY INCUBATION
WITH 10⁻⁹M GROWTH HORMONE OVER 0-60 MINUTES

Hepatocytes pretreated with 10⁻⁸M dexamethasone showed a marked stimulation of all enzyme activities (Table 17) as was shown in Figure 19. These dexamethasone-exposed hepatocytes were then incubated with 10⁻⁹M growth hormone over a further 0-60 minutes prior to being assayed for steroid-metabolising activity (Table 18).

Figure 20 shows that incubation with 10⁻⁹M growth hormone markedly reduced 7 α -hydroxylase activity to basal levels at all time points as compared to the response obtained with dexamethasone alone.

FIGURE 19 : Effects of preincubation with 10^{-8} M DEXAMETHASONE over 24, 48 and 72 hours on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 17



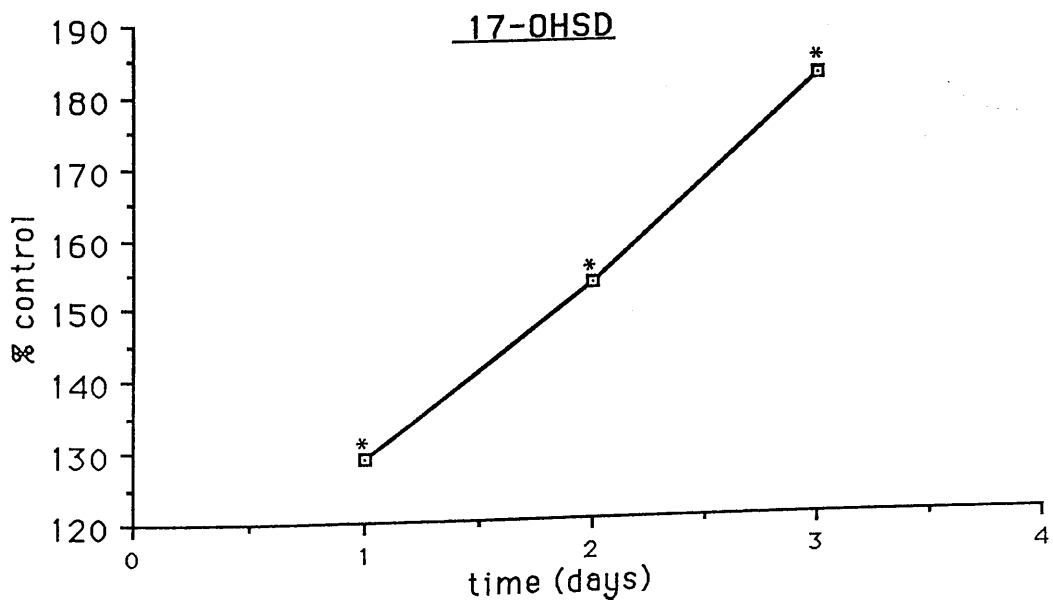
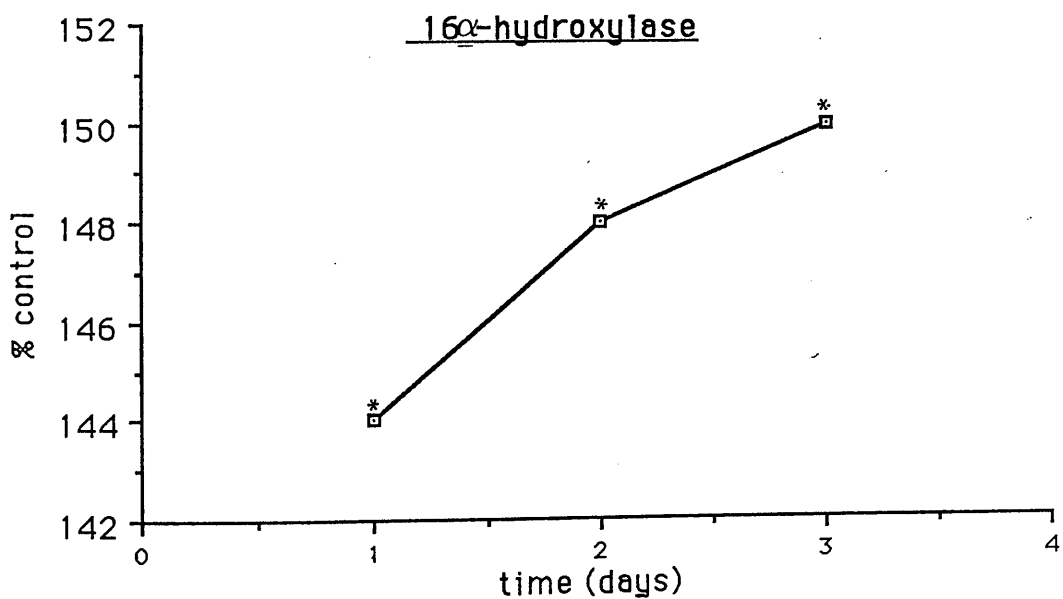
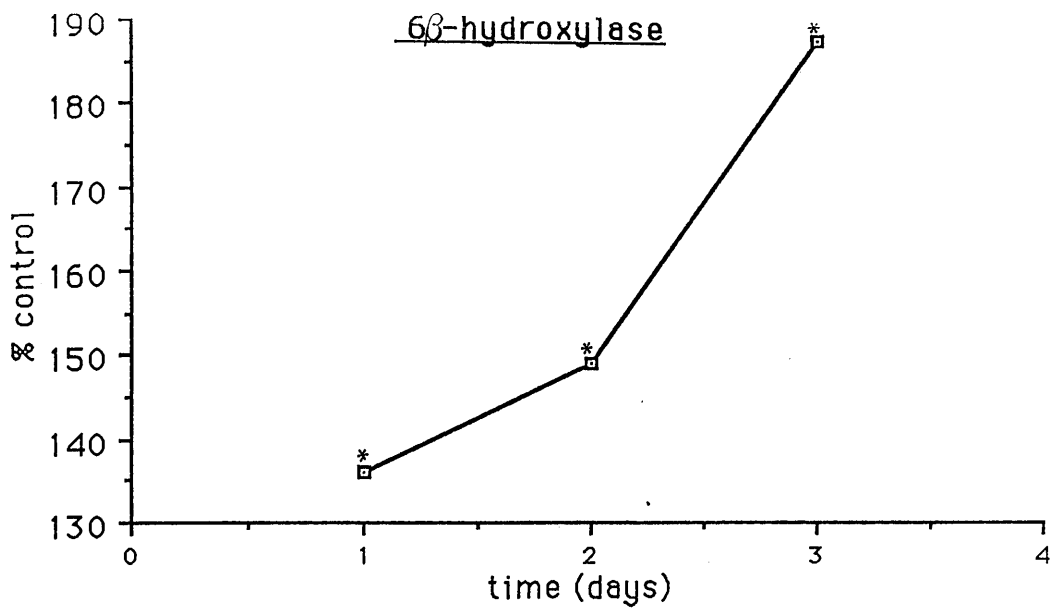


TABLE 17 : Effects of preincubation with 10^{-8} M DEX over 24,48 and 72h on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

<u>TREATMENT</u>	<u>Enzyme activities (pmoles/minute/million cells)</u>				
	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
24 C	42 \pm 3	44 \pm 1	32 \pm 1	34 \pm 4	116 \pm 5
24 DEX	76 \pm 7*	60 \pm 6*	46 \pm 1*	44 \pm 1*	152 \pm 1*
48 C	46 \pm 3	48 \pm 2	80 \pm 2	52 \pm 5	123 \pm 9
48 DEX	80 \pm 3*	72 \pm 2*	118 \pm 2*	80 \pm 6*	200 \pm 7*
72 C	28 \pm 3	36 \pm 6	28 \pm 1	22 \pm 2	94 \pm 7
72 DEX	50 \pm 2*	68 \pm 3*	42 \pm 2*	40 \pm 2*	181 \pm 9*

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values (C=control).

FIGURE 20 : Effects of preincubation with 10^{-8} M DEXAMETHASONE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 7 α -hydroxylase activity. Results are expressed as percentages of relevant control values, where $\ast = P < 0.05$ and $N = 3$. Absolute data is given in Table 18

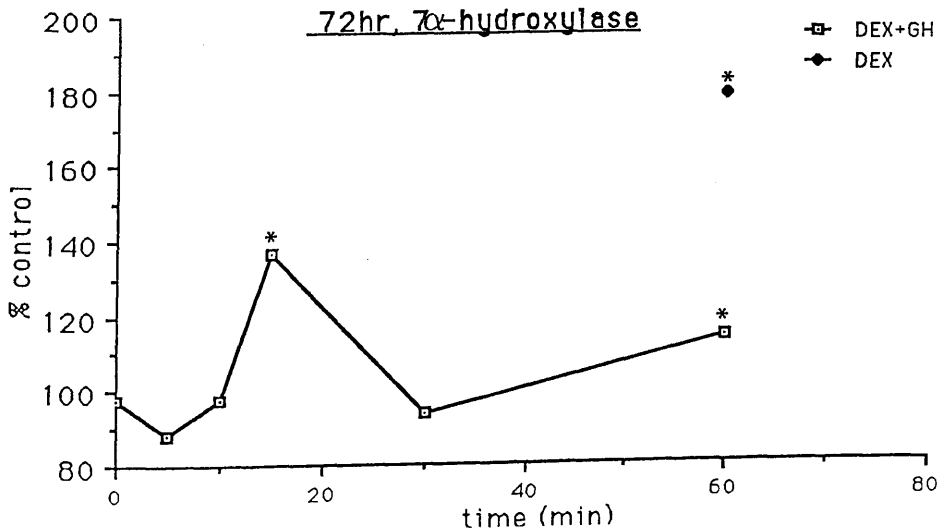
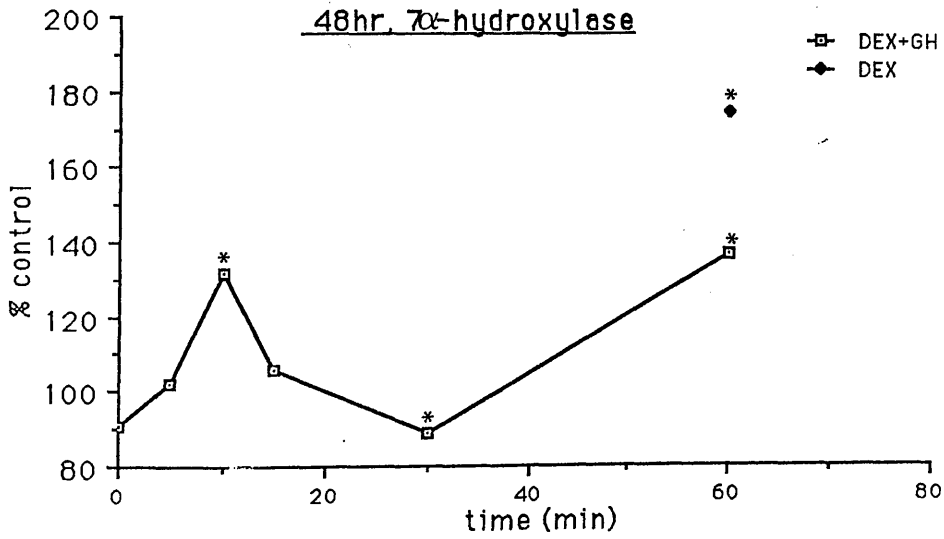
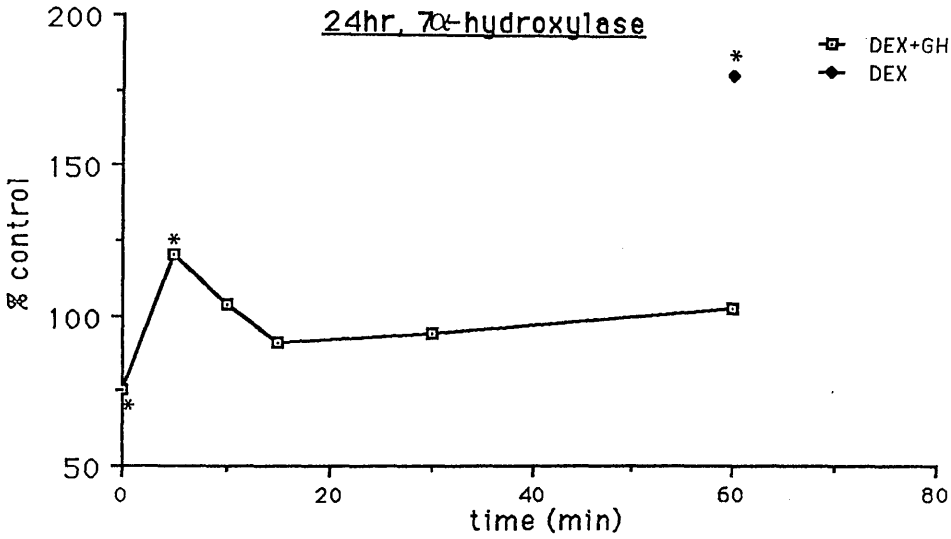


FIGURE 21 : Effects of preincubation with 10^{-8} M DEXAMETHASONE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 6β -hydroxylase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 18

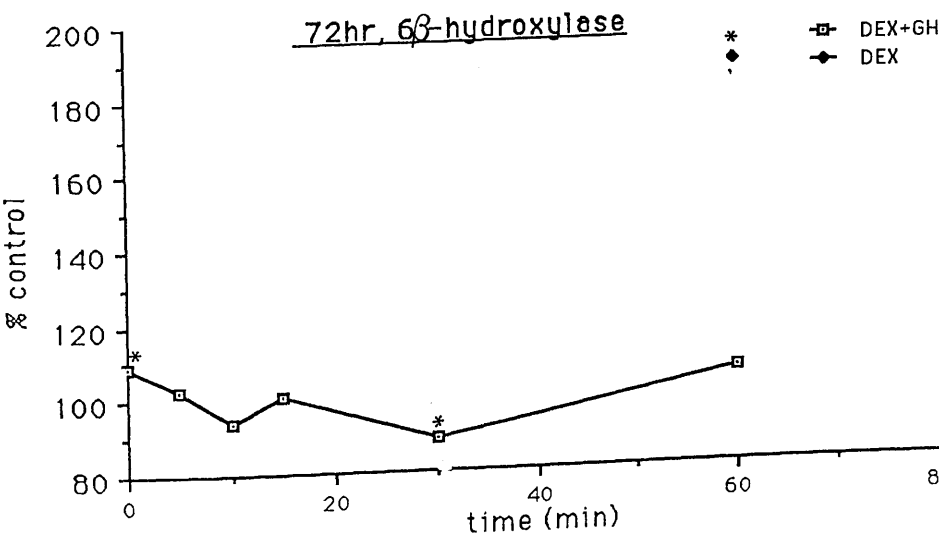
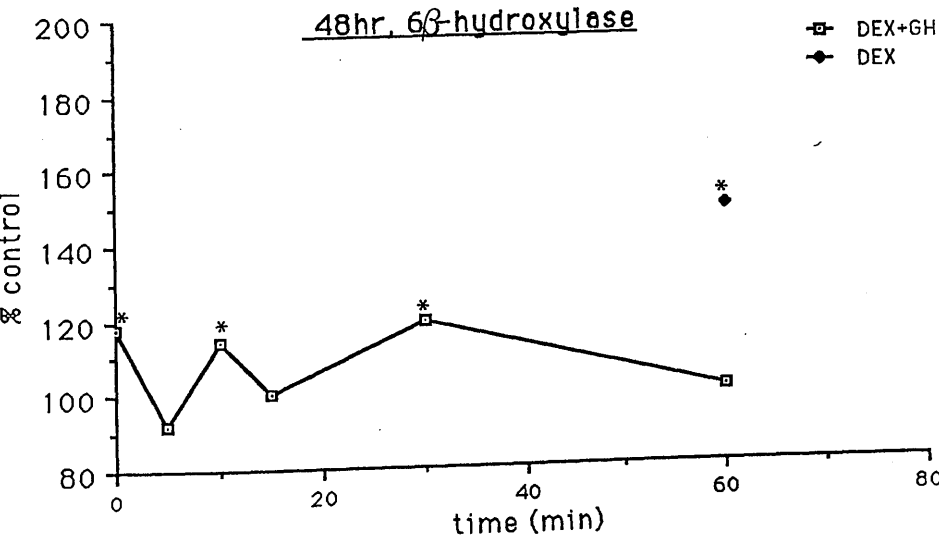
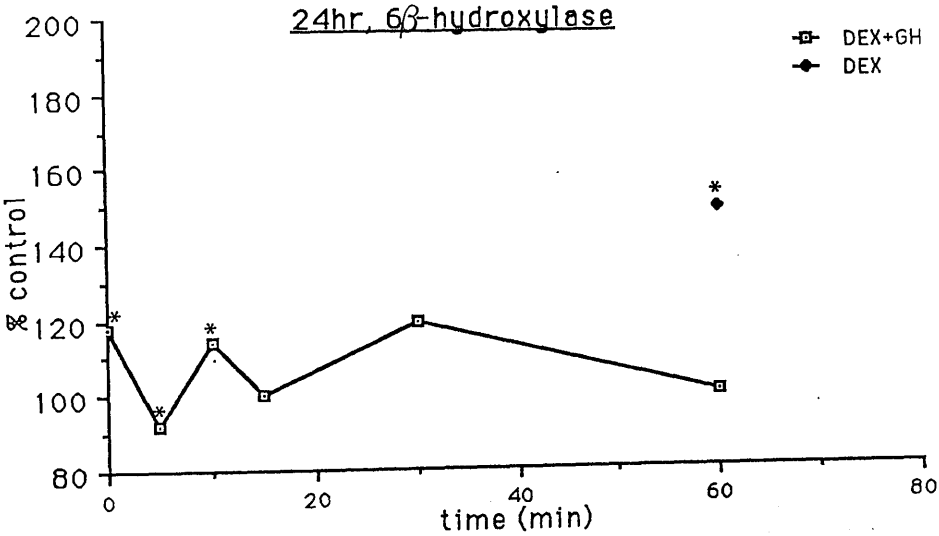


FIGURE 22 : Effects of preincubation with 10^{-8} M DEXAMETHASONE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 16α -hydroxylase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 18

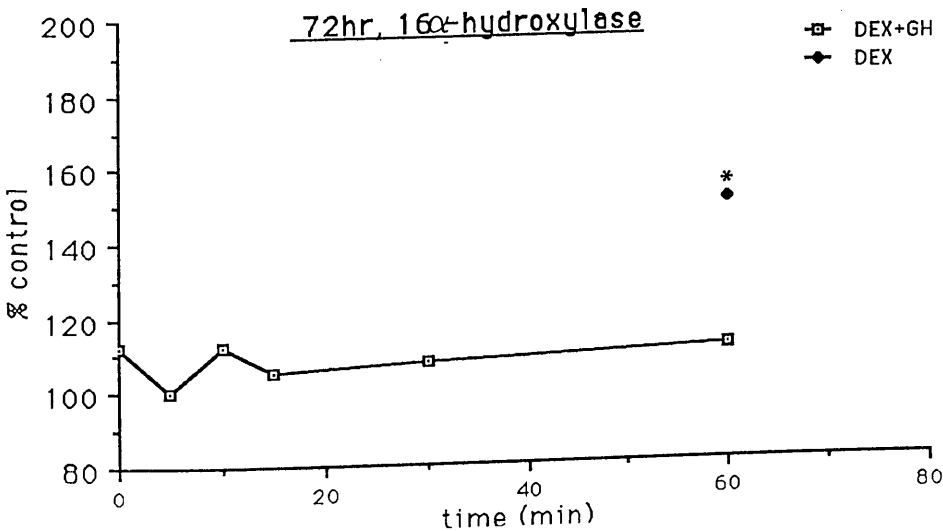
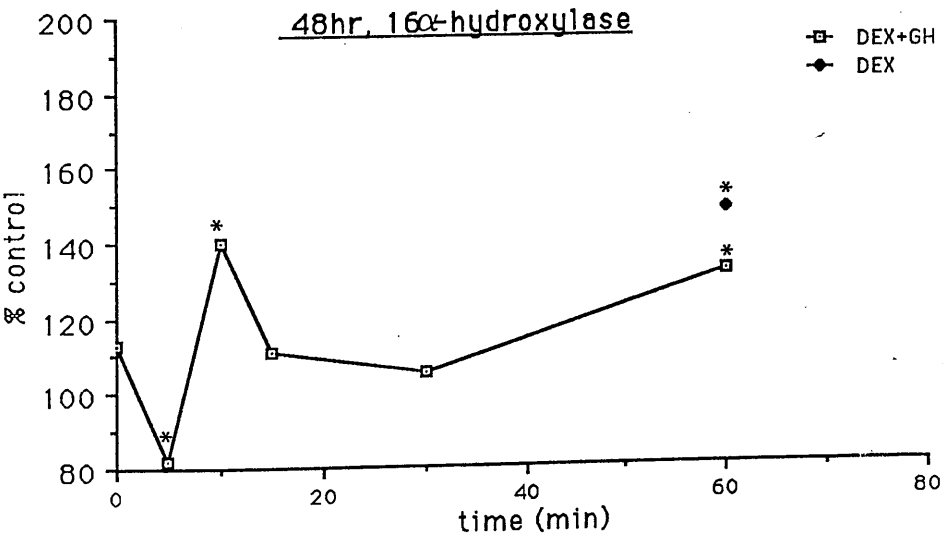
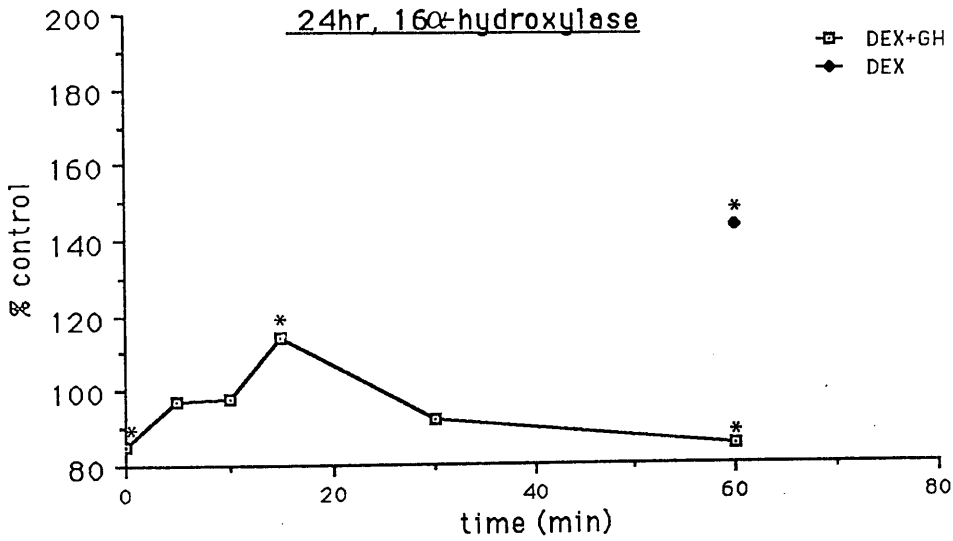


FIGURE 23 : Effects of preincubation with 10^{-8} M DEXAMETHASONE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 17-oxosteroid oxidoreductase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 18.

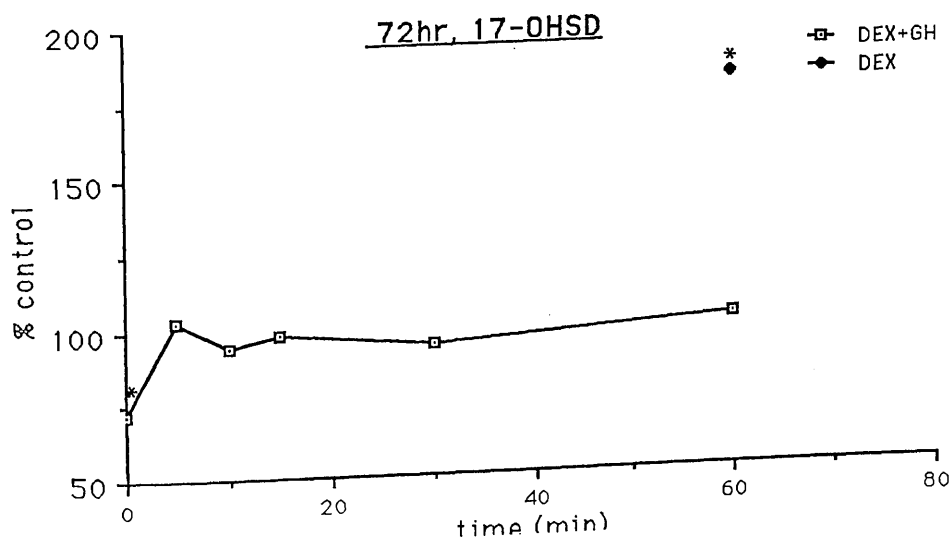
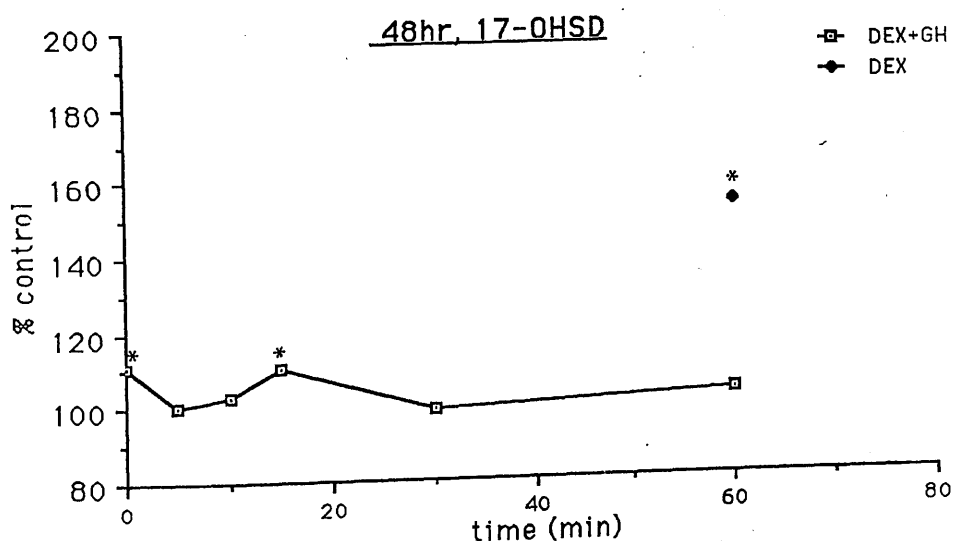
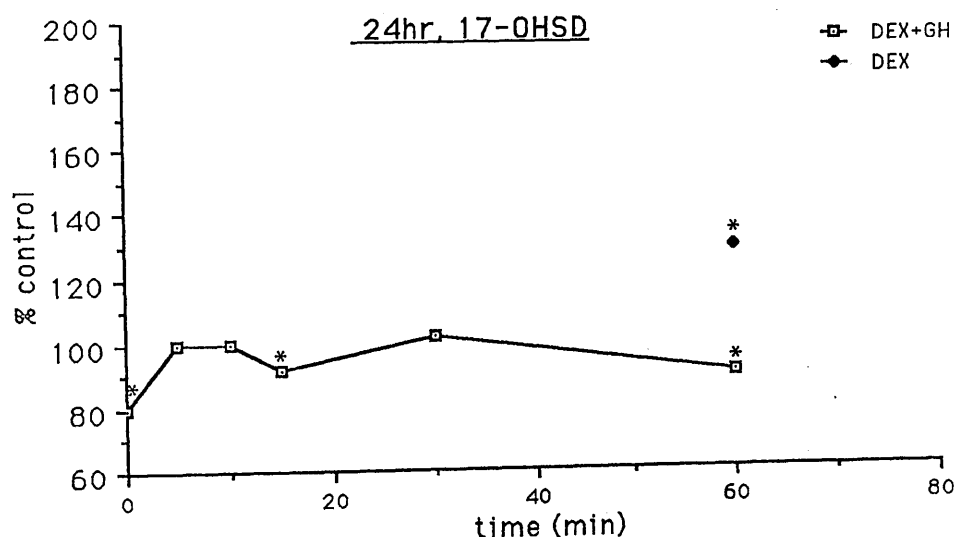


FIGURE 24 : Effects of preincubation with 10^{-8} M DEXAMETHASONE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 5 α -reductase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 18

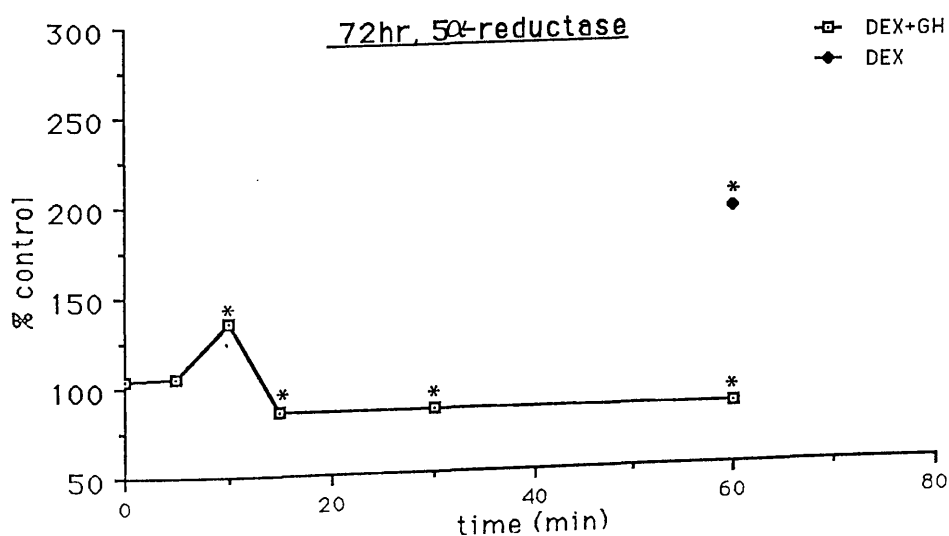
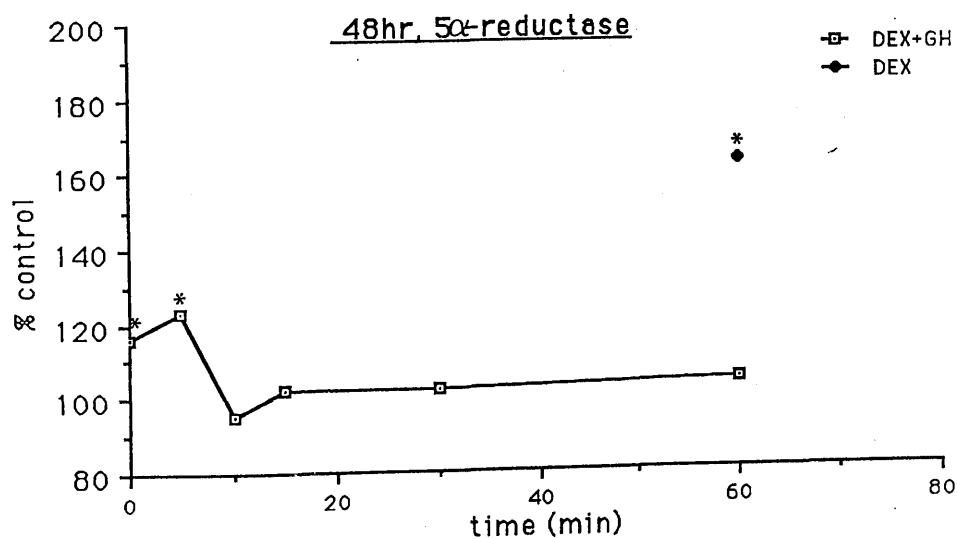
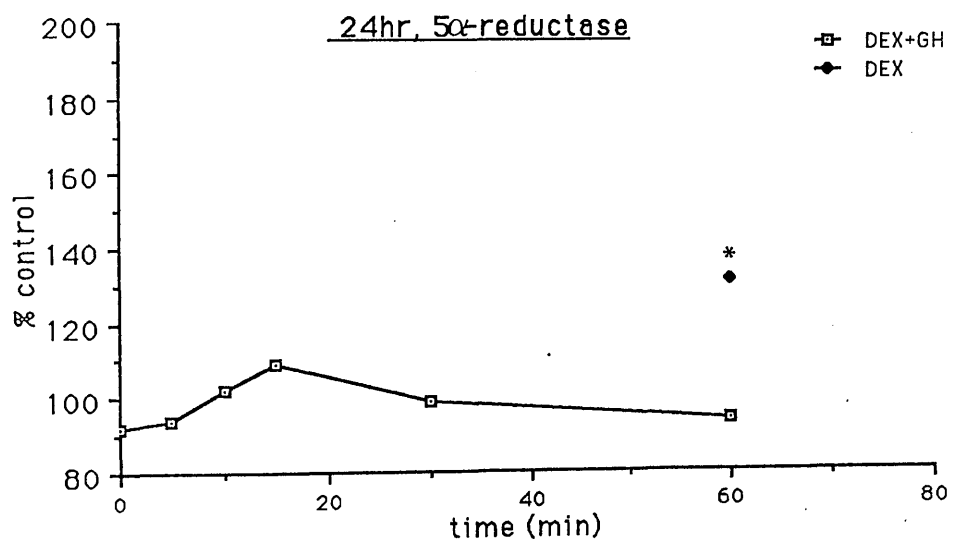
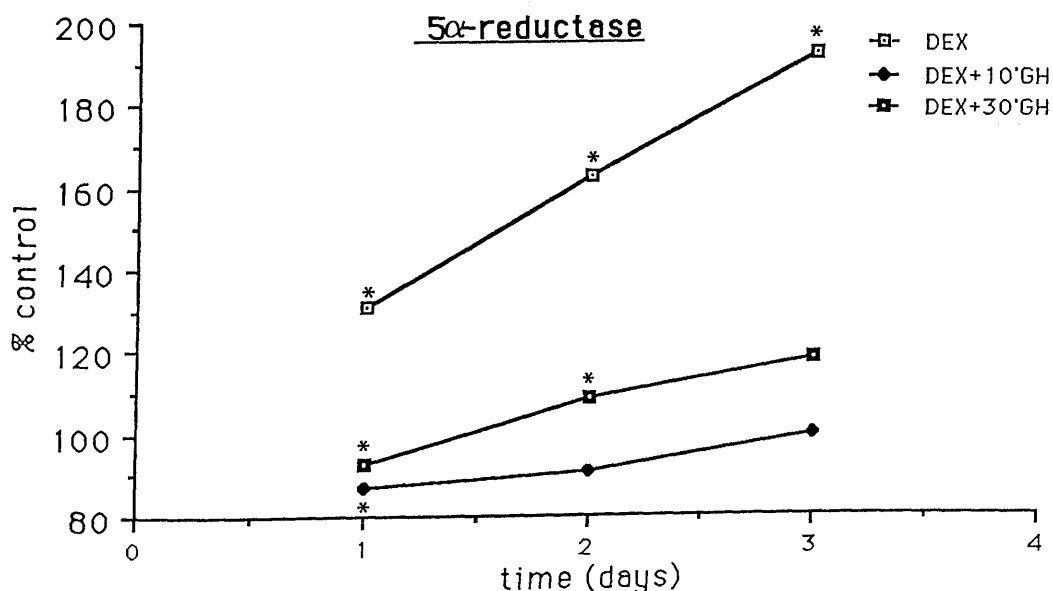
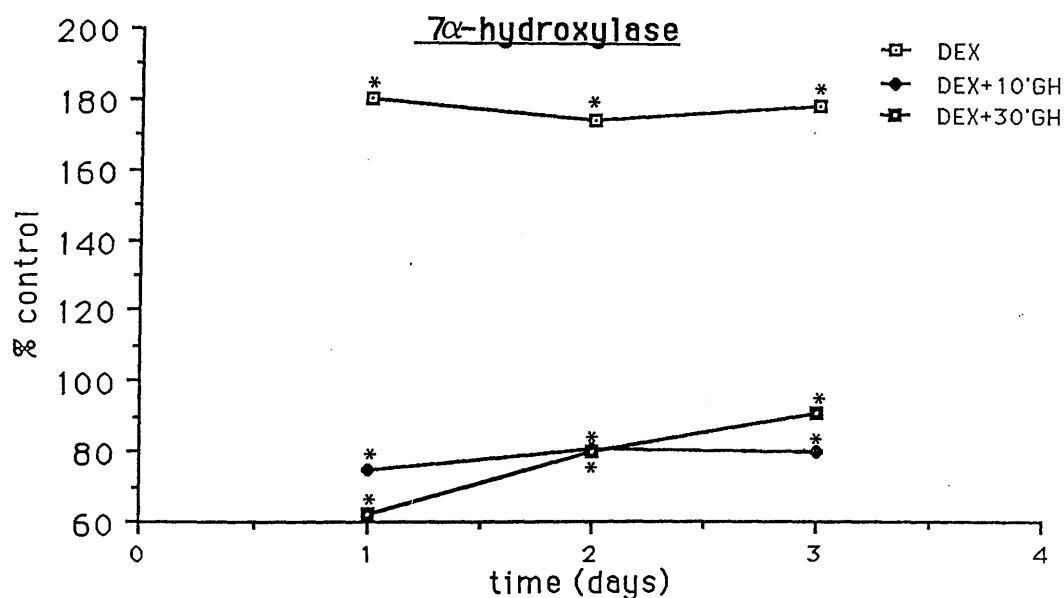


FIGURE 25 : Effects of preincubation with 10^{-8} M DEXAMETHASONE over 24, 48 and 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE for 10 and 30 minutes on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17-oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 18



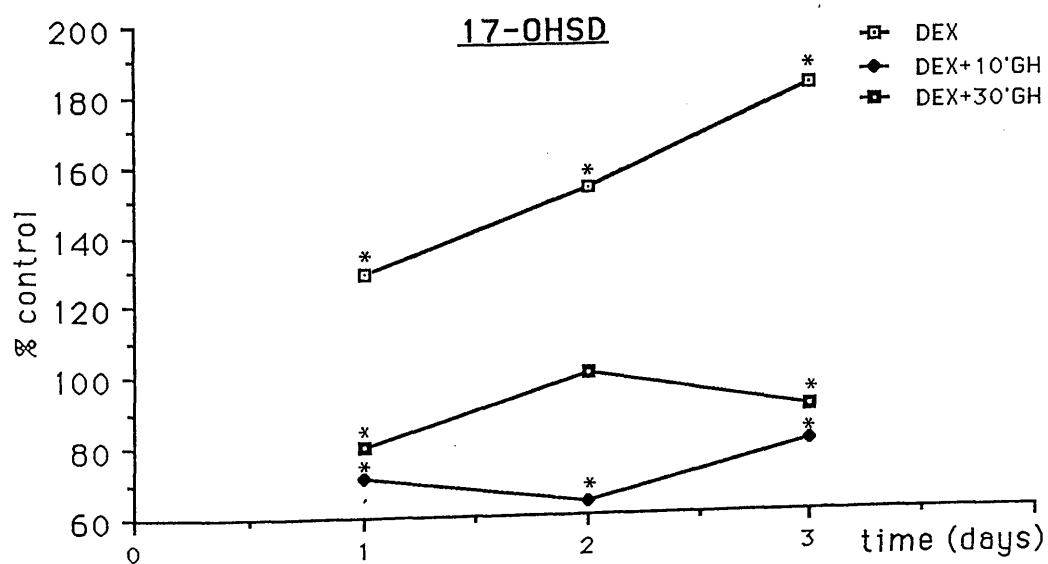
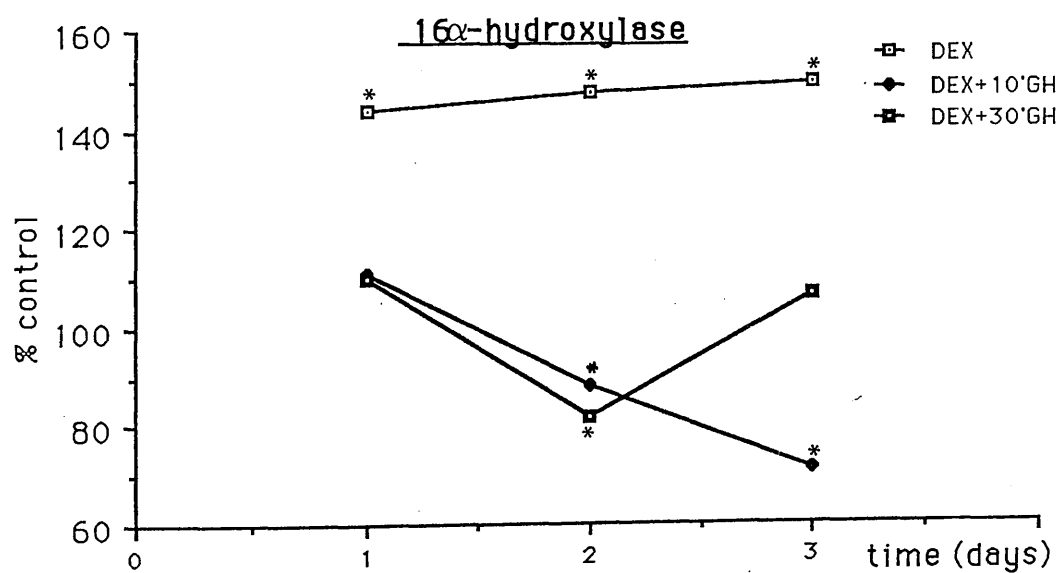
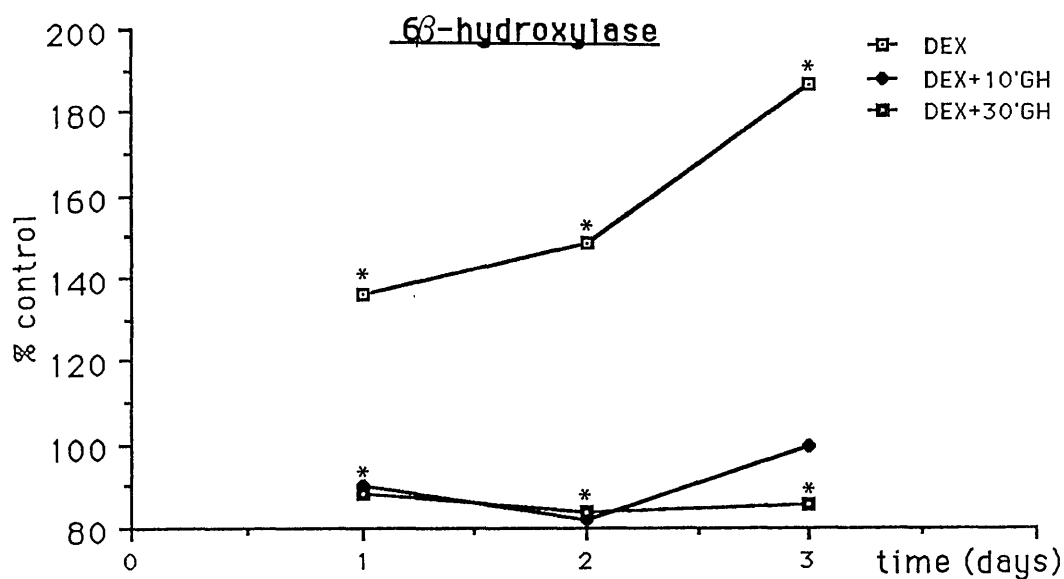


TABLE 18 : Effects of preincubation with 10^{-8} M DEX over 24,48 and 72h followed by incubation with 10^{-9} M GROWTH HORMONE for 0 to 60 minutes of A) 24h-pretreated, B) 48h-pretreated and C) 72h-pretreated hepatocytes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

A)	Enzyme activities (pmoles/minute/million cells)				
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
24h C	33 \pm 3	35 \pm 3	32 \pm 3	80 \pm 7	117 \pm 6
24h DEX	60 \pm 5*	48 \pm 3*	46 \pm 3*	103 \pm 6*	153 \pm 7*
control	39 \pm 4	49 \pm 2	33 \pm 2	118 \pm 4	126 \pm 2
24DEX+0GH	28 \pm 3*	36 \pm 2*	28 \pm 2*	94 \pm 9*	116 \pm 9
control	23 \pm 2	33 \pm 1	30 \pm 2	86 \pm 4	124 \pm 5
24DEX+5GH	28 \pm 2*	36 \pm 2*	28 \pm 3	86 \pm 2	116 \pm 5
control	26 \pm 2	39 \pm 4	27 \pm 4	87 \pm 1	112 \pm 6
24DEX+10GH	27 \pm 1	33 \pm 2*	26 \pm 2	87 \pm 5	114 \pm 2
control	28 \pm 4	34 \pm 1	24 \pm 1	94 \pm 4	106 \pm 4
24DEX+15GH	25 \pm 2	35 \pm 3	28 \pm 2*	87 \pm 1*	116 \pm 10
control	25 \pm 2	34 \pm 1	29 \pm 3	86 \pm 1	120 \pm 4
24DEX+30GH	23 \pm 3	35 \pm 1	26 \pm 3	88 \pm 5	119 \pm 2
control	29 \pm 1	32 \pm 1	34 \pm 3	94 \pm 2	121 \pm 5
24DEX+60GH	30 \pm 3	32 \pm 3	28 \pm 2*	85 \pm 4*	114 \pm 8

B)	Enzyme activities (pmoles/minute/million cells)				
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
48h C	34 \pm 1	44 \pm 3	29 \pm 2	96 \pm 7	133 \pm 13
48h DEX	60 \pm 4*	66 \pm 4*	43 \pm 4*	147 \pm 7*	217 \pm 17*
control	28 \pm 5	33 \pm 3	28 \pm 3	86 \pm 1	118 \pm 10
48DEX+0GH	26 \pm 2	39 \pm 2*	31 \pm 2	96 \pm 2*	137 \pm 12*
control	25 \pm 2	40 \pm 2	32 \pm 1	99 \pm 1	118 \pm 6
48DEX+5GH	26 \pm 2	37 \pm 1	26 \pm 2*	98 \pm 1	145 \pm 15*
control	26 \pm 2	38 \pm 1	29 \pm 2	87 \pm 8	134 \pm 6
48DEX+10GH	35 \pm 3*	44 \pm 3*	40 \pm 6*	90 \pm 7	127 \pm 5
control	26 \pm 3	34 \pm 2	27 \pm 2	88 \pm 2	128 \pm 5
48DEX+15GH	28 \pm 1	34 \pm 2	30 \pm 3	98 \pm 5*	130 \pm 6
control	31 \pm 1	34 \pm 2	28 \pm 2	88 \pm 4	125 \pm 7
48DEX+30GH	28 \pm 1*	40 \pm 1*	30 \pm 3	87 \pm 4	127 \pm 4
control	27 \pm 4	41 \pm 1	30 \pm 1	90 \pm 3	128 \pm 10
48DEX+60GH	37 \pm 1*	41 \pm 2	39 \pm 2*	92 \pm 6	134 \pm 10

C)	Enzyme activities (pmoles/minute/million cells)				
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
72h C	27 \pm 3	34 \pm 2	32 \pm 3	80 \pm 4	109 \pm 4
72h DEX	48 \pm 5*	64 \pm 5*	48 \pm 3*	146 \pm 10*	210 \pm 11*
control	31 \pm 1	32 \pm 2	26 \pm 1	80 \pm 2	114 \pm 4
72DEX+0GH	30 \pm 1	35 \pm 1*	28 \pm 3	57 \pm 6*	82 \pm 6
control	29 \pm 3	31 \pm 2	24 \pm 1	76 \pm 5	115 \pm 12
72DEX+5GH	25 \pm 2	32 \pm 3	24 \pm 1	78 \pm 1	80 \pm 3
control	26 \pm 1	36 \pm 2	26 \pm 2	83 \pm 6	112 \pm 5
72DEX+10GH	26 \pm 3	35 \pm 2	28 \pm 1	78 \pm 3	101 \pm 7*
control	23 \pm 1	35 \pm 1	28 \pm 2	86 \pm 7	116 \pm 12
72DEX+15GH	32 \pm 3*	36 \pm 2	29 \pm 2	84 \pm 6	66 \pm 3*
control	28 \pm 1	40 \pm 1	26 \pm 1	93 \pm 2	123 \pm 14
72DEX+30GH	26 \pm 1	35 \pm 2*	28 \pm 2	87 \pm 5	69 \pm 4*
control	22 \pm 2	30 \pm 2	24 \pm 1	80 \pm 2	123 \pm 14
72DEX+60GH	25 \pm 1*	32 \pm 2	26 \pm 3	80 \pm 1	69 \pm 3*

Results are expressed as mean \pm s.d. (N=3); *= P <0.05 as compared to relevant control values (C=control).

The effects of growth hormone on 6 β -hydroxylase (Figure 21) were more marked with cells pretreated with dexamethasone for 72h, although a reduction was also observed with hepatocytes pretreated for 24 and 48h. Again this reduction in enzyme activity to basal levels was apparent early in the time course. Similarly, incubation of dexamethasone-pretreated hepatocytes with growth hormone resulted in a marked decrease in 16 α -hydroxylase (Figure 22), 17-oxosteroid oxidoreductase (Figure 23) and 5 α -reductase activities (Figure 24) throughout the 60 minute time course.

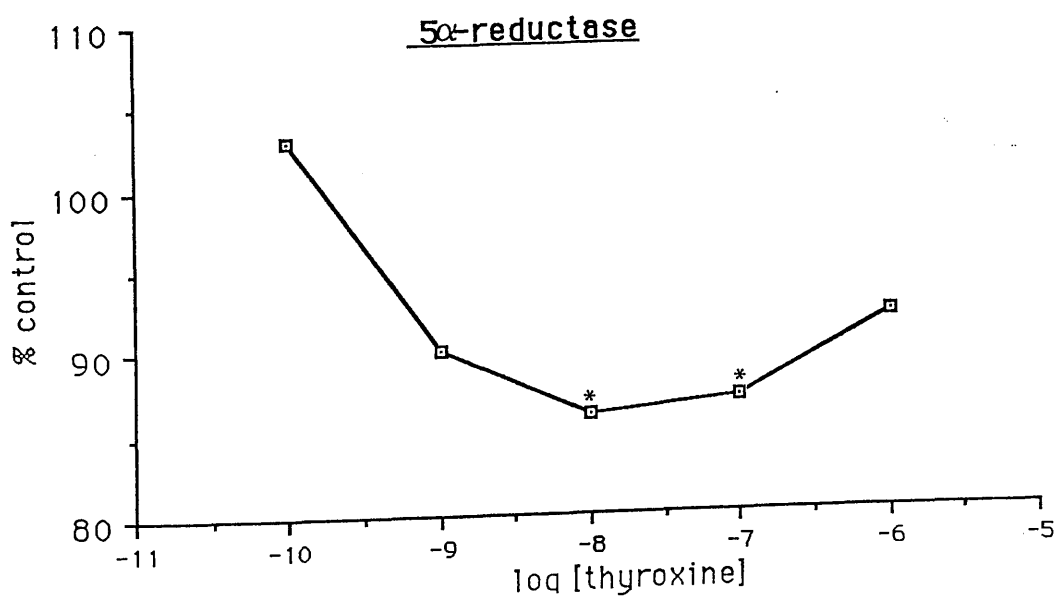
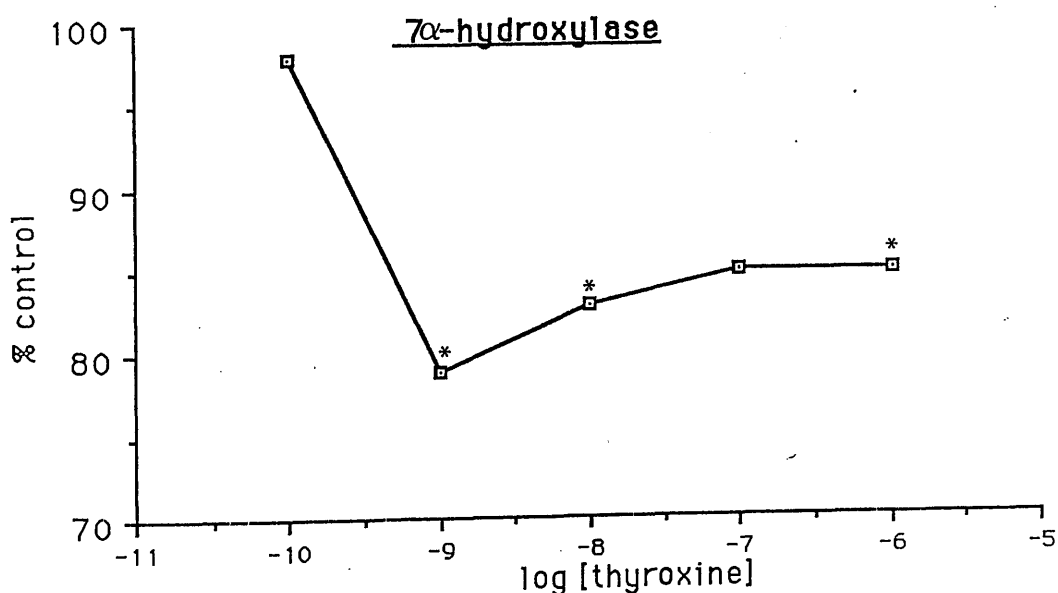
Figure 25 summarises the above results. Thus the potent stimulatory effects of dexamethasone on these enzymes are strongly modulated by further incubation with growth hormone in a non-specific manner, as was observed with similar experiments over short incubation periods (section 3.3.1).

3.4 THYROXINE

3.4.1 DOSE-DEPENDENT EFFECTS OF THYROXINE

Hepatocytes were preincubated with 10^{-10} M- 10^{-6} M thyroxine for 10 and 30 minutes (Tables 19A and 19B respectively). Incubation with 10^{-10} M thyroxine for 10 minutes caused no significant changes in the female-specific activities 7 α -hydroxylase (Fig 26A) and 5 α -reductase (Figure 26B), but significantly increased the male-specific 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid

FIGURE 26 : Effects of 10 minute preincubation with 10^{-10} M- 10^{-6} M THYROXINE on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 19A



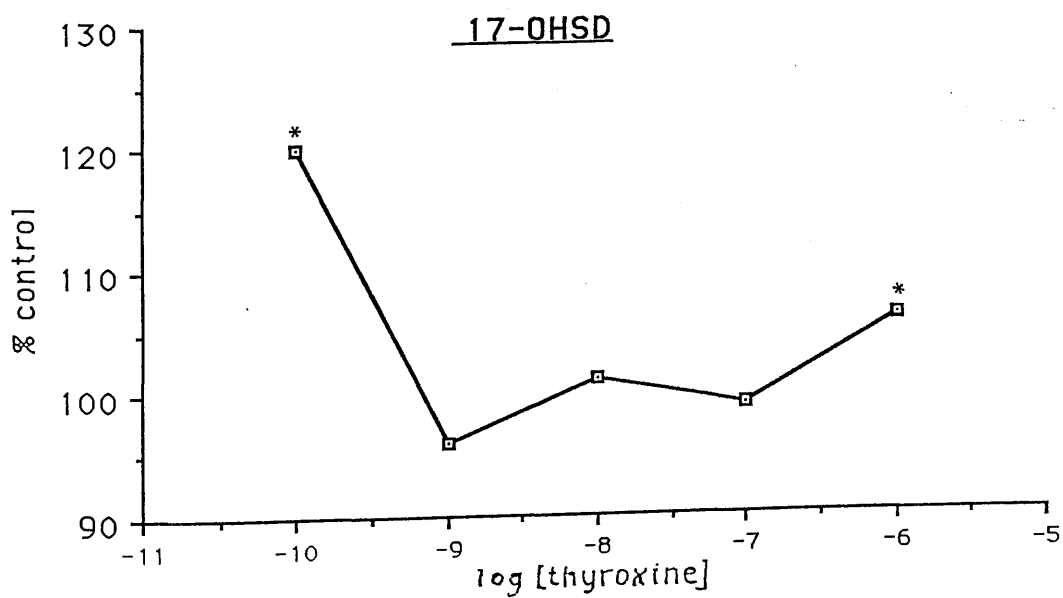
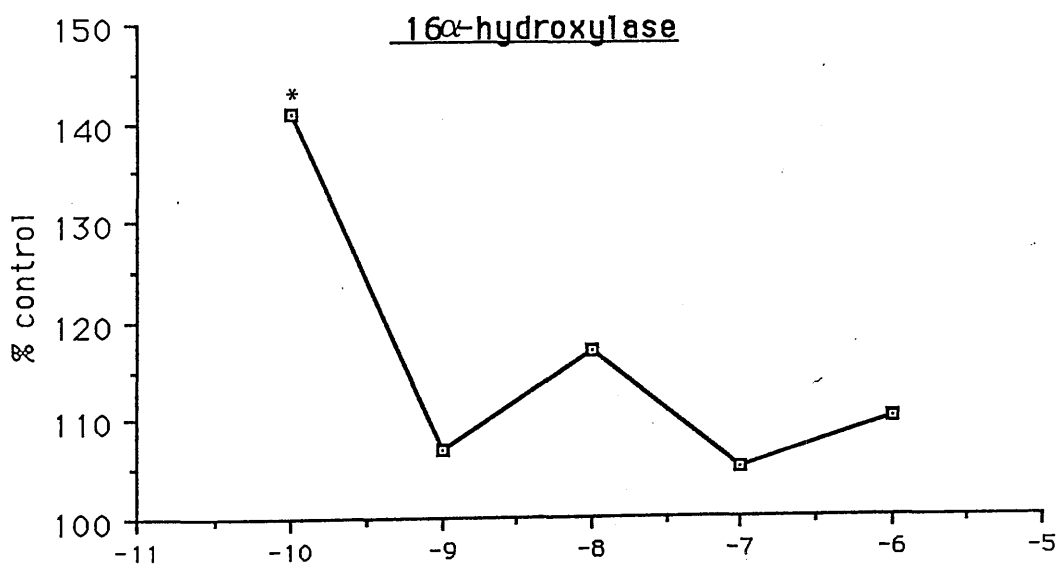
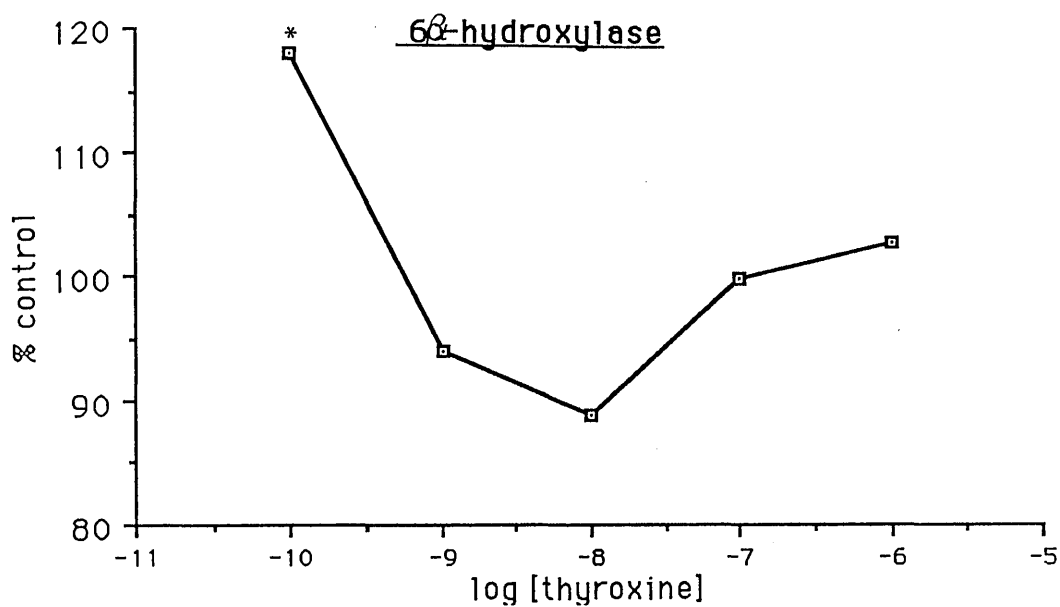
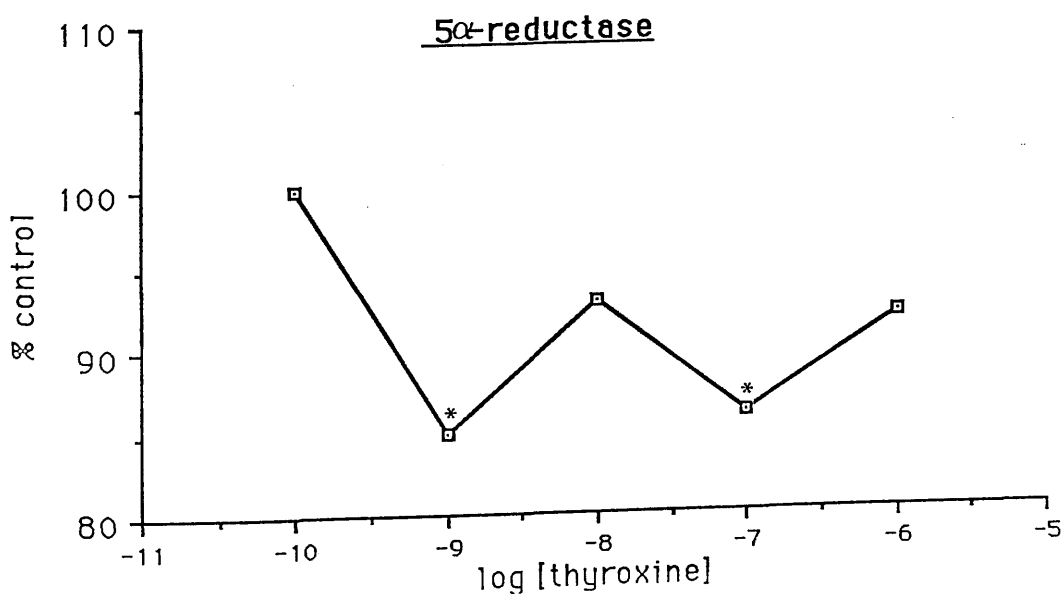
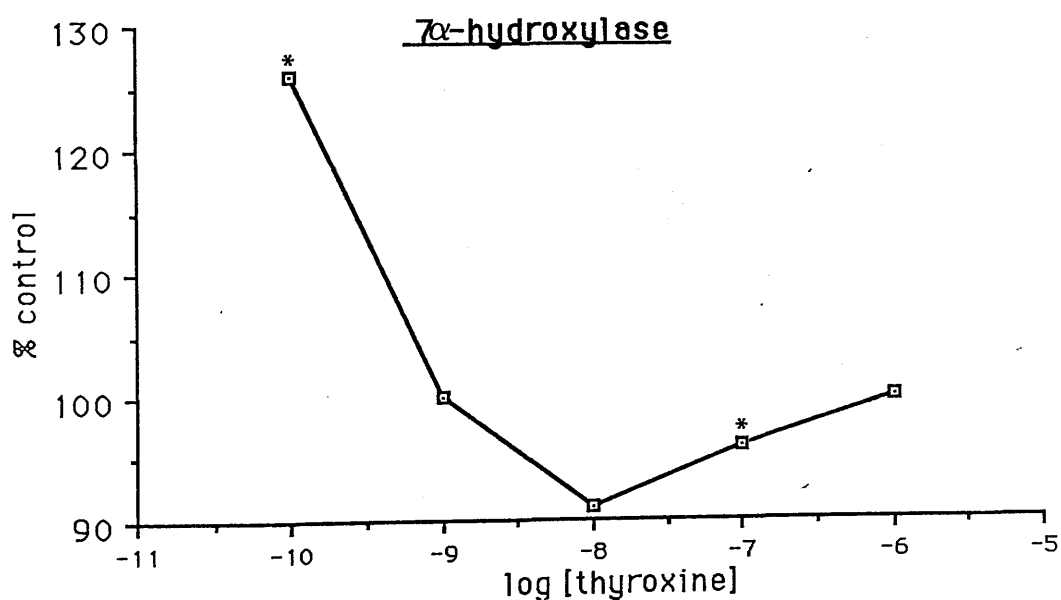


FIGURE 27 : Effects of 30 minute preincubation with 10^{-10}M - 10^{-6}M THYROXINE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 19B



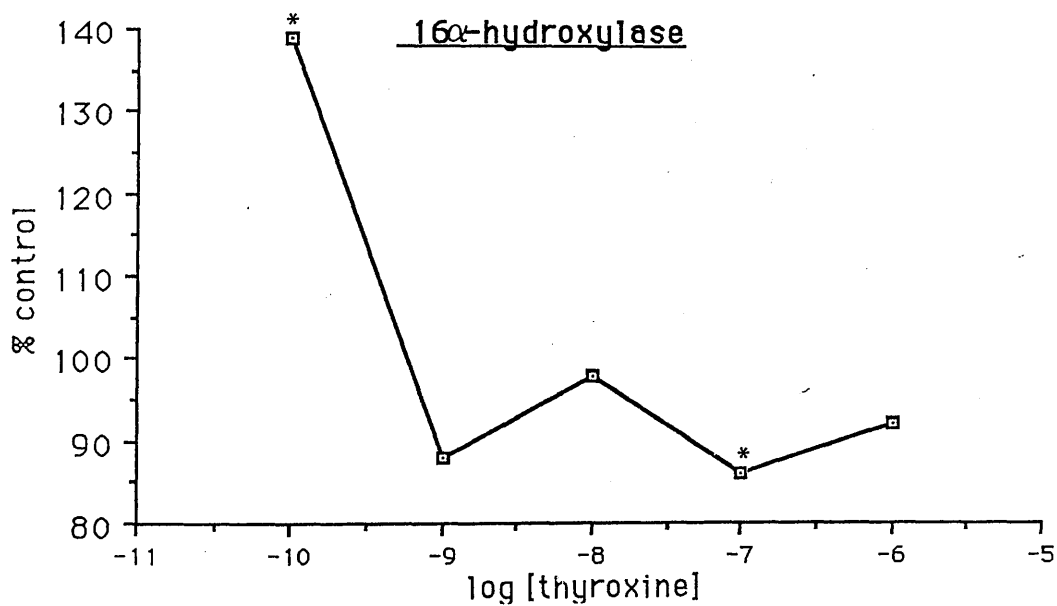
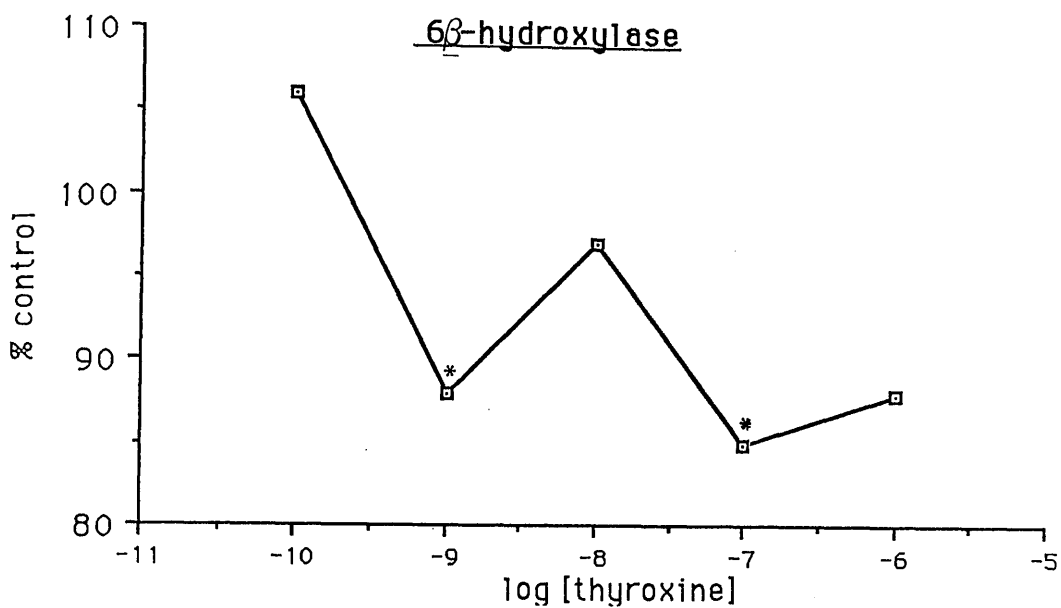


TABLE 19 : Effects of preincubation with 10^{-10}M - 10^{-6}M THYROXINE for A) 10 and B) 30 minutes on 7α -, 6β -, 16α -hydroxylases (OHases), 17-oxosteroid oxidoreductase and 5α -reductase activities.

A) Enzyme activities (pmoles/minute/million cells)

TREATMENT	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
Control (A)	65 \pm 7	63 \pm 7	42 \pm 5	138 \pm 4	122 \pm 13
10^{-10}M (B)	64 \pm 4	74 \pm 5*	59 \pm 2*	166 \pm 8*	126 \pm 6
10^{-9}M (C)	51 \pm 2*	59 \pm 4	45 \pm 5	133 \pm 5	110 \pm 2
10^{-8}M (D)	54 \pm 6*	56 \pm 3	49 \pm 5	139 \pm 1	105 \pm 4*
10^{-7}M (E)	55 \pm 6	63 \pm 6	44 \pm 4	137 \pm 6	106 \pm 1*
10^{-6}M (F)	55 \pm 2*	65 \pm 2	46 \pm 6	146 \pm 3*	112 \pm 12

B) Enzyme activities (pmoles/minute/million cells)

TREATMENT	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
Control (A)	57 \pm 1	67 \pm 5	49 \pm 4	137 \pm 3	114 \pm 6
10^{-10}M (B)	72 \pm 2*	71 \pm 2	68 \pm 6*	148 \pm 6*	114 \pm 5
10^{-9}M (C)	57 \pm 8	59 \pm 2*	48 \pm 2	124 \pm 14	97 \pm 8*
10^{-8}M (D)	52 \pm 7	65 \pm 3	48 \pm 6	133 \pm 2	107 \pm 4
10^{-7}M (E)	55 \pm 1*	57 \pm 2*	42 \pm 2*	123 \pm 10*	98 \pm 10*
10^{-6}M (F)	57 \pm 6	59 \pm 7	45 \pm 6	137 \pm 4	105 \pm 10

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values.

Duncan's Multiple Range Test :

A)

7α -OHase	<u>C D E F B A</u>
6β -OHase	<u>D C A E F B</u>
16α -OHase	<u>A E C F D B</u>
17-OHSD	<u>C E A D F B</u>
5α -red	<u>D E C F A B</u>

B)

7α -OHase	<u>D E A C F B</u>
6β -OHase	<u>E C F D A B</u>
16α -OHase	<u>E F C D A B</u>
17-OHSD	<u>E C D A F B</u>
5α -red	<u>C E F D A B</u>

oxidoreductase activities (Figures 26C, 26D and 26E respectively). Incubation with higher concentrations of thyroxine significantly decreased female-specific activities (Figures 26A and 26B) whilst exhibiting no significant effects upon male-specific enzymes (Figures 26C, 26D and 26E). Thus thyroxine appears to exhibit sex-specific effects at 10 minutes of incubation.

Incubation of hepatocytes with the above doses of thyroxine for 30 minutes (Table 19B) resulted in a similar response to that observed at 10 minutes above.

7 α -hydroxylase activity (Figure 27A) was significantly increased with 10^{-10} M thyroxine, thereafter significantly decreased to 90% of control with 10^{-8} M thyroxine.

5 α -reductase activity (Figure 27B) was significantly decreased with all thyroxine concentrations used, except 10^{-10} M, where enzyme activity remained unchanged.

Male-specific activities (Figures 27C, 27D, 27E) were significantly increased with 10^{-10} M thyroxine, after which point all activities returned to basal levels or below, and reached a significant reduction with 10^{-7} M thyroxine in all cases.

Thus incubation with thyroxine appears to stimulate male-specific activities at low concentrations and reduced all activities at higher concentrations, thereby exhibiting some sex-specific effects on steroid metabolism.

3.4.2 COMPARISON OF THE INTERACTION OF VARYING CONCENTRATIONS OF THYROXINE WITH 10^{-9} M GROWTH HORMONE

Incubation of hepatocytes with 10^{-8} M thyroxine (Table 20) for 30 minutes resulted in no significant changes in enzyme activity (Figure 28). Incubation with 10^{-6} M thyroxine resulted in significant decreases in 6β -hydroxylase, 16α -hydroxylase and 5α -reductase activities, whilst having no effects on either 7α -hydroxylase or 17-oxosteroid oxidoreductase activities (Figure 28).

Co-incubation with growth hormone resulted in significant reductions in enzyme activity in all cases, as is illustrated in Figure 28, except for in 7α -hydroxylase and 5α -reductase activities with 10^{-8} M thyroxine.

3.4.3 TIME COURSE OF THE INTERACTION BETWEEN THYROXINE AND GROWTH HORMONE

Hepatocytes were preincubated with 10^{-6} M thyroxine with or without 10^{-9} M growth hormone for 10-30 minutes (Table 21). Incubation with thyroxine alone resulted in significant reductions in 6β -hydroxylase, 17-oxosteroid oxidoreductase and 5α -reductase activities (Figures 29C, 29E and 29B respectively). Co-incubation with 10^{-9} M growth hormone resulted in marked reductions in all enzyme activities (Figure 29). The decrease in enzyme activity is non-specific in nature since all activities were reduced upon incubation with growth hormone and thyroxine.

FIGURE 28 : A comparison of the effects of 30 minute preincubation with 10^{-8} M or 10^{-6} M THYROXINE in the absence and presence of 10^{-9} M GROWTH HORMONE on enzyme activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 20

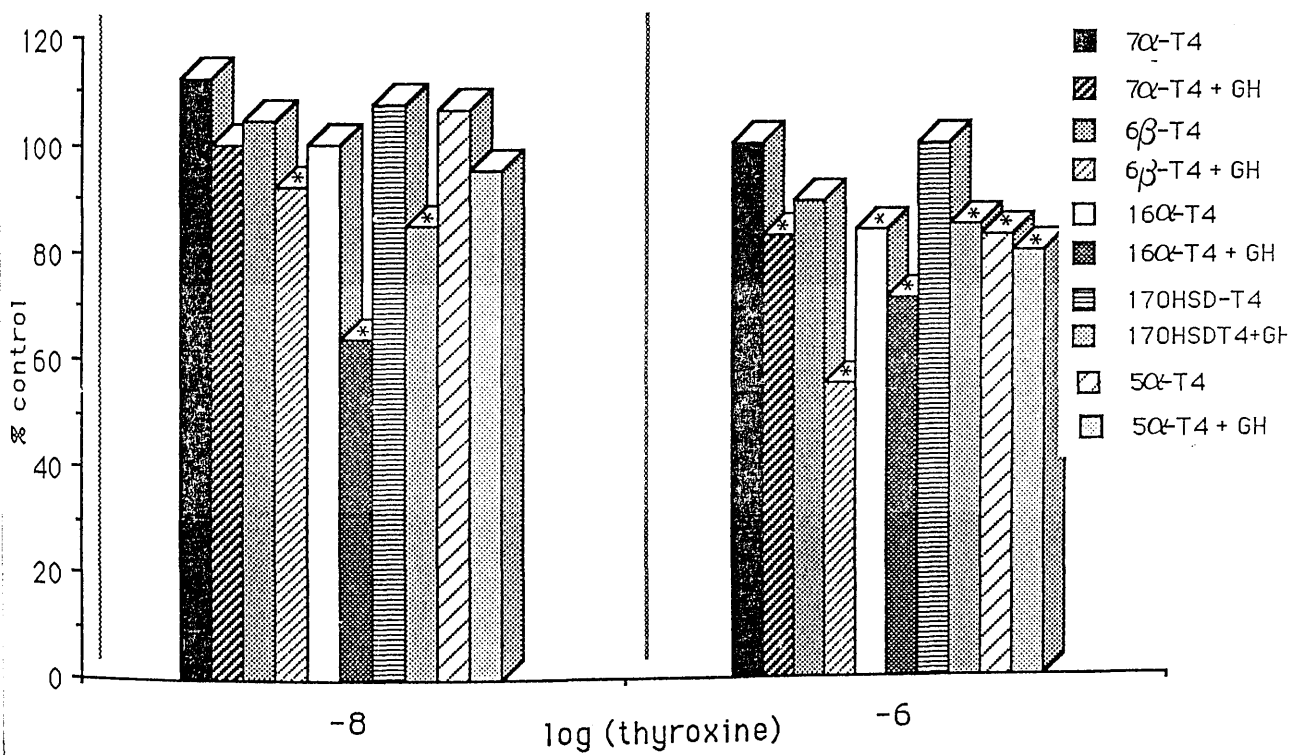
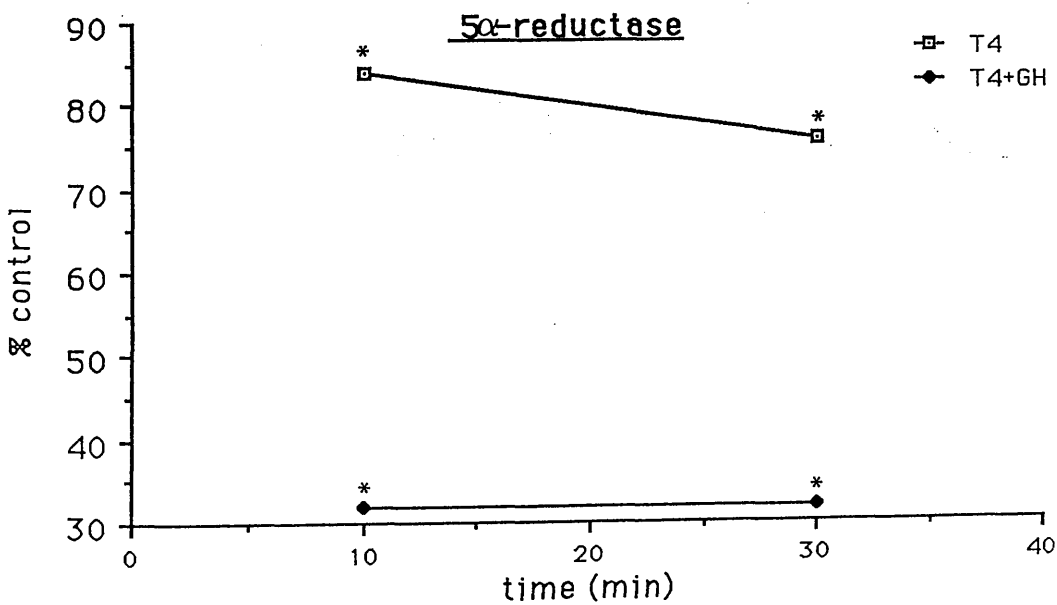
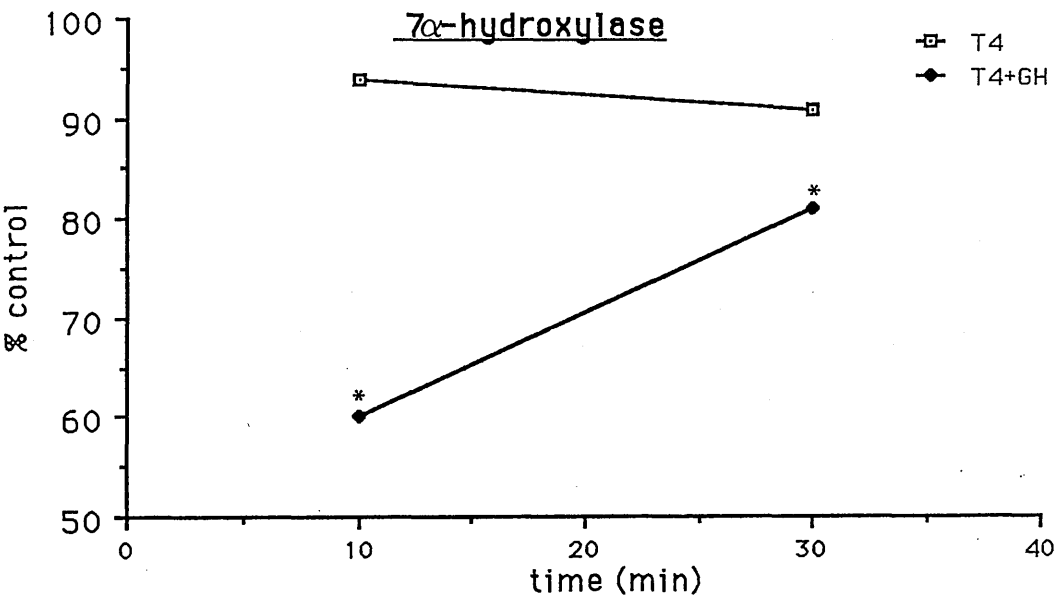


TABLE 20 : Effects of 30 minute preincubation with 10^{-8} M or 10^{-6} M THYROXINE in the absence and presence of 10^{-9} M GROWTH HORMONE on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

<u>Enzyme activities (pmoles/minute/million cells)</u>					
<u>TREATMENT</u>	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
Control	32 \pm 2	56 \pm 6	50 \pm 2	30 \pm 1	108 \pm 11
-8 T ₄	36 \pm 4	58 \pm 4	50 \pm 3	32 \pm 1	114 \pm 14
-6 T ₄	32 \pm 1	50 \pm 1	42 \pm 5*	30 \pm 2	90 \pm 1*
Control	36 \pm 1	76 \pm 4	56 \pm 4	52 \pm 1	102 \pm 11
-8 T ₄ + GH	36 \pm 2	70 \pm 2*	36 \pm 1*	44 \pm 1*	97 \pm 6
-6 T ₄ + GH	30 \pm 2*	42 \pm 2*	40 \pm 1*	44 \pm 1*	82 \pm 9*

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values.

FIGURE 29 : Effects of preincubation with 10^{-6} M THYROXINE in the absence and presence of 10^{-9} M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 21



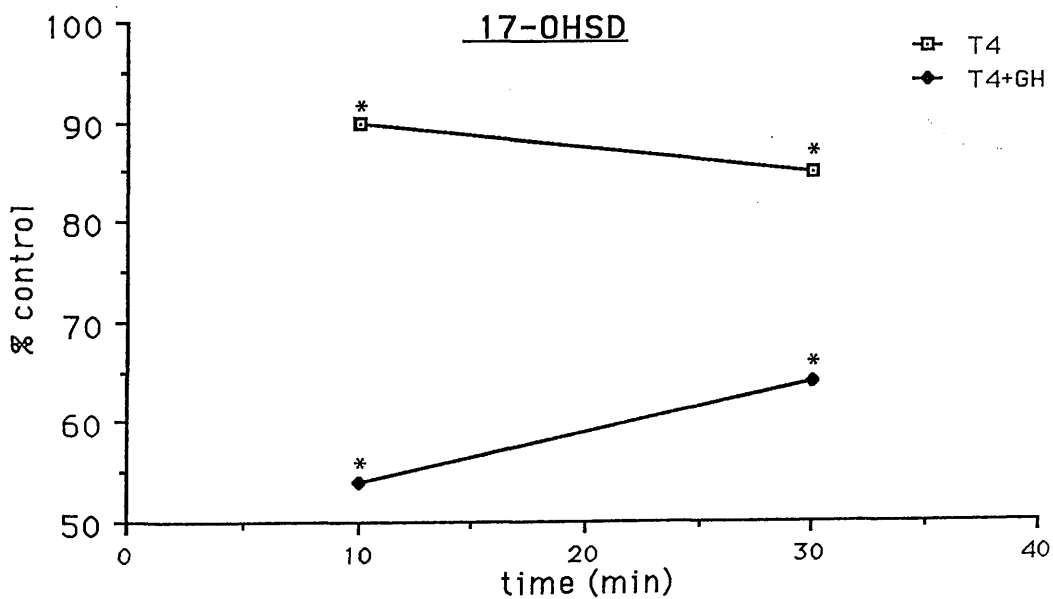
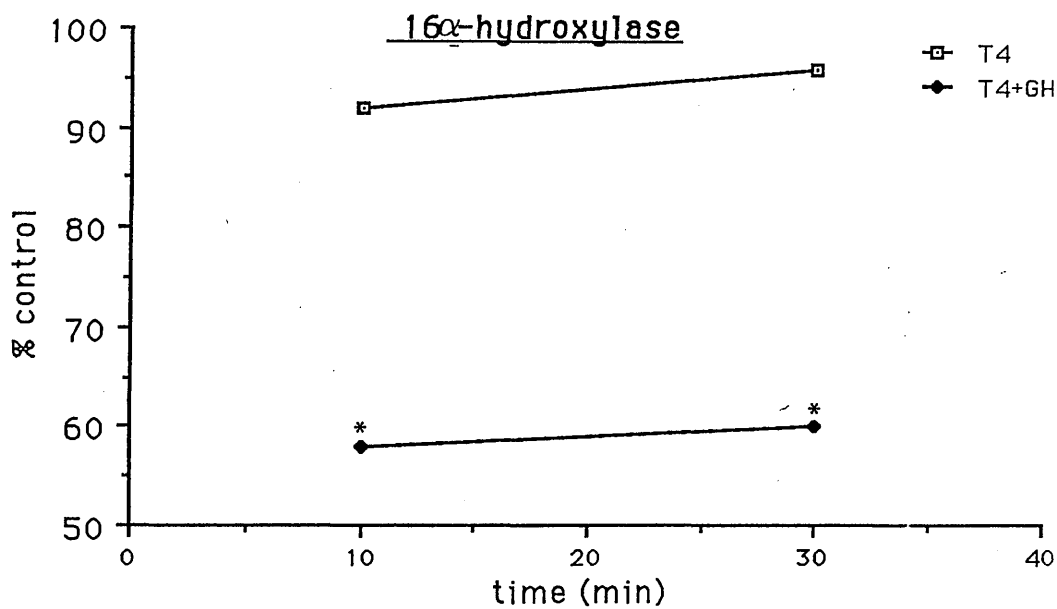
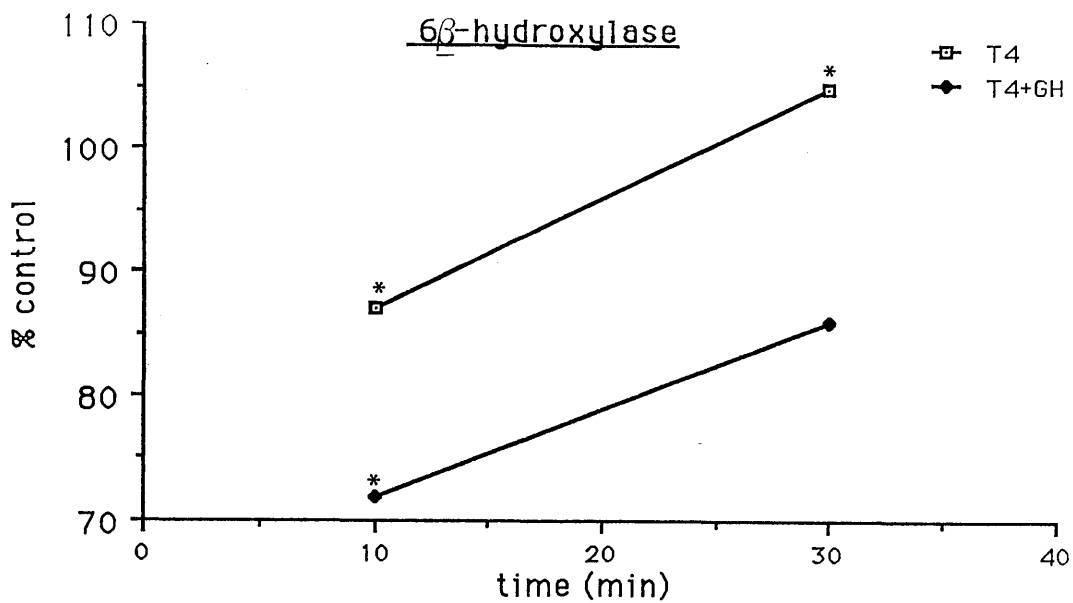


TABLE 21 : Effects of preincubation with 10^{-6} M THYROXINE in the absence or presence of 10^{-9} M GROWTH HORMONE for 10 and 30 minutes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

<u>TREATMENT</u>	<u>Enzyme activities (pmoles/minute/million cells)</u>				
	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
10 C	42 \pm 4	61 \pm 6	76 \pm 6	117 \pm 1	117 \pm 6
10 T ₄	39 \pm 5	53 \pm 1*	70 \pm 5	105 \pm 2*	98 \pm 6*
30 C	45 \pm 4	59 \pm 2	71 \pm 6	109 \pm 5	126 \pm 7
30 T ₄	41 \pm 4	62 \pm 1*	68 \pm 7	93 \pm 1*	96 \pm 5*
10 C	45 \pm 6	60 \pm 3	77 \pm 4	128 \pm 6	130 \pm 12
10 T ₄ + GH	27 \pm 3*	43 \pm 2*	45 \pm 8*	69 \pm 6*	42 \pm 8*
30 C	51 \pm 2	56 \pm 7	82 \pm 9	124 \pm 4	142 \pm 16
30 T ₄ + GH	41 \pm 5*	48 \pm 2	49 \pm 6*	79 \pm 4*	45 \pm 2*

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values (C=control).

3.4.4 PREINCUBATION OF HEPATOCYTES WITH THYROXINE

(10^{-6} M) FOR 24, 48 AND 72 HOURS FOLLOWED BY INCUBATION
WITH 10^{-9} M GROWTH HORMONE OVER 0 TO 60 MINUTES.

Hepatocytes were preincubated with 10^{-6} M thyroxine for 24, 48 and 72 hours, followed by further incubation with 10^{-9} M growth hormone for 0-60 minutes, as detailed in Section 2.5.2.

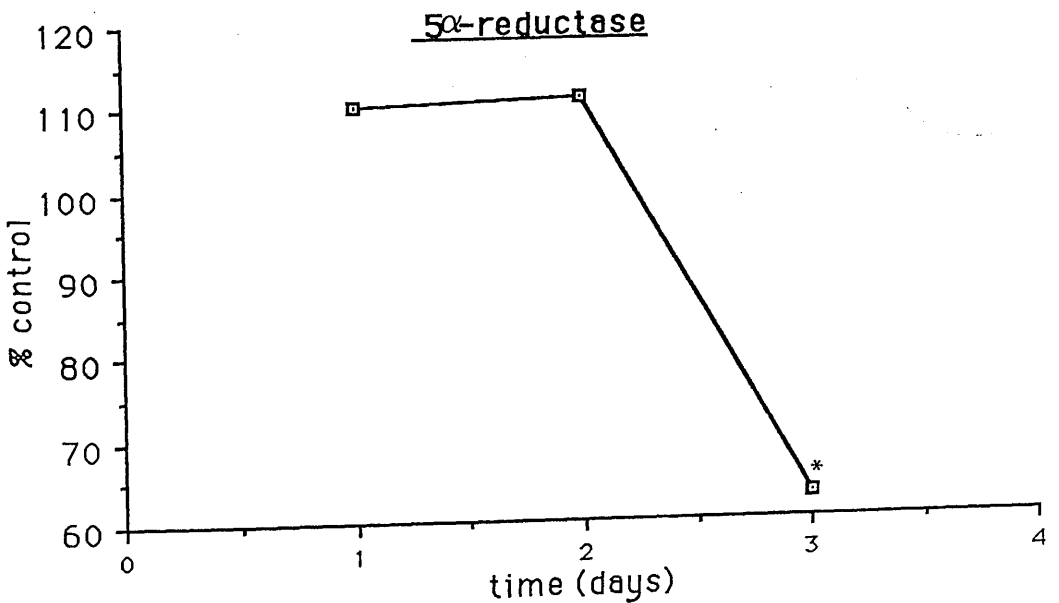
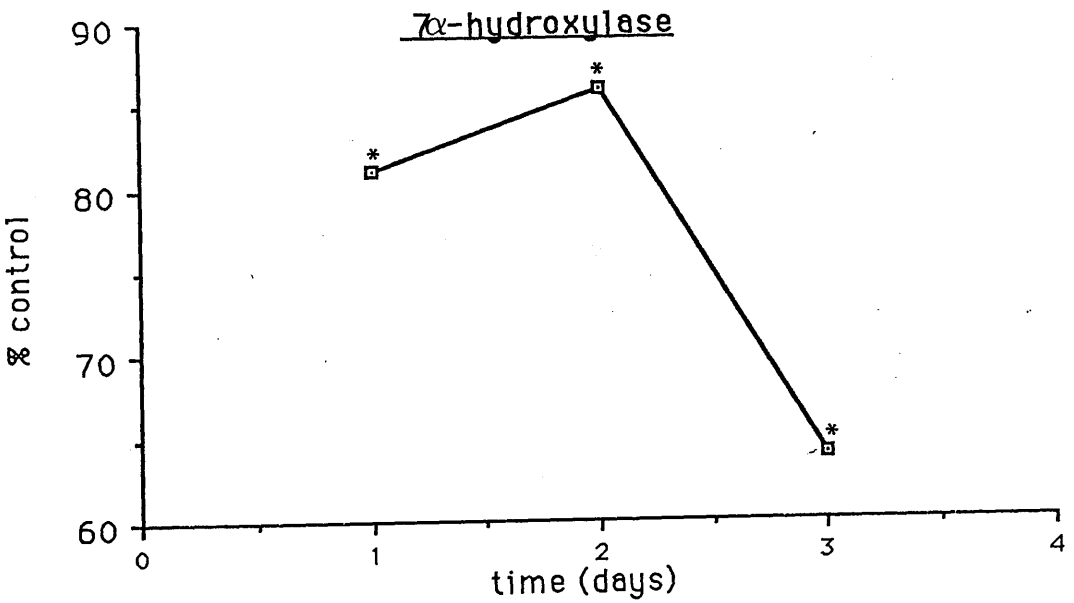
Preincubation with thyroxine for (Table 22) showed no significant effects on enzyme activity after 24 and 48 hours of culture (Figure 30), except for a decrease in 7 α -hydroxylase (Figure 30A), and an increase in 6 β -hydroxylase (Figure 30C) activities. All enzyme activities were significantly reduced at 72h (Figure 30) as compared to relevant control values.

Further incubation with 10^{-9} M growth hormone for 0 to 60 minutes (Table 22) show that enzyme activity was modulated upon treatment with growth hormone. 7 α -hydroxylase activity (Figure 31) was markedly increased as compared to the thyroxine alone response, being restored to basal levels as compared to relevant control incubations.

6 β -hydroxylase activity remained relatively unchanged upon incubation with growth hormone after 24 and 48 hours of culture, but increased to near basal levels with hepatocytes pretreated with thyroxine for 72 hours (Figure 32).

Similar results were observed with 16 α -hydroxylase, 17-oxosteroid oxidoreductase and 5 α -reductase activities (Figures 33, 34 and 35 respectively) as with 6 β -hydroxylase above.

FIGURE 30 : Effects of preincubation with 10^{-6} M THYROXINE over 24, 48 and 72 hours on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*$ = $P<0.05$ and $N=3$. Absolute data is given in Table 22



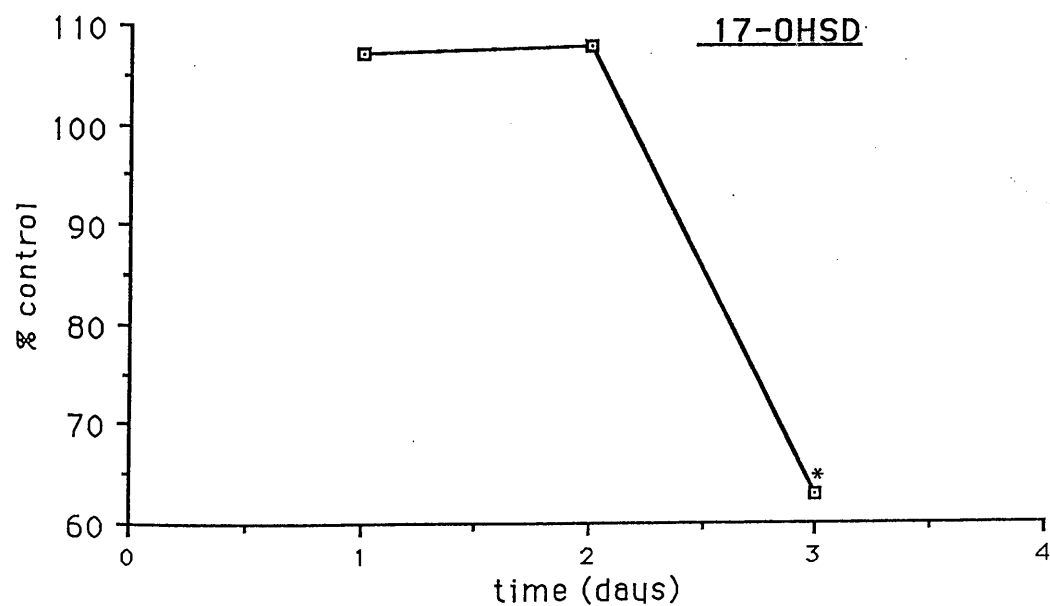
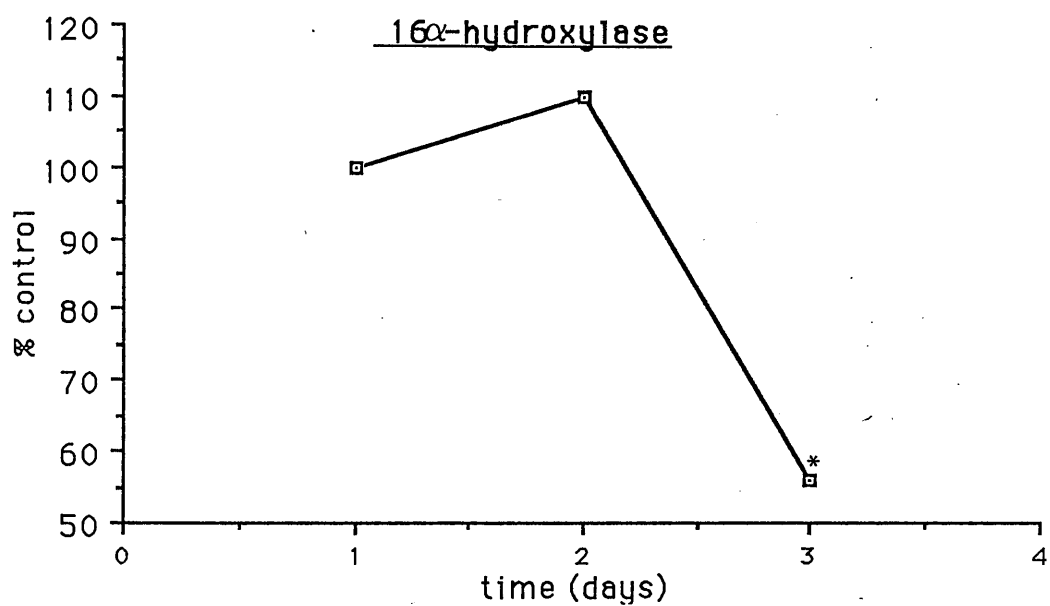
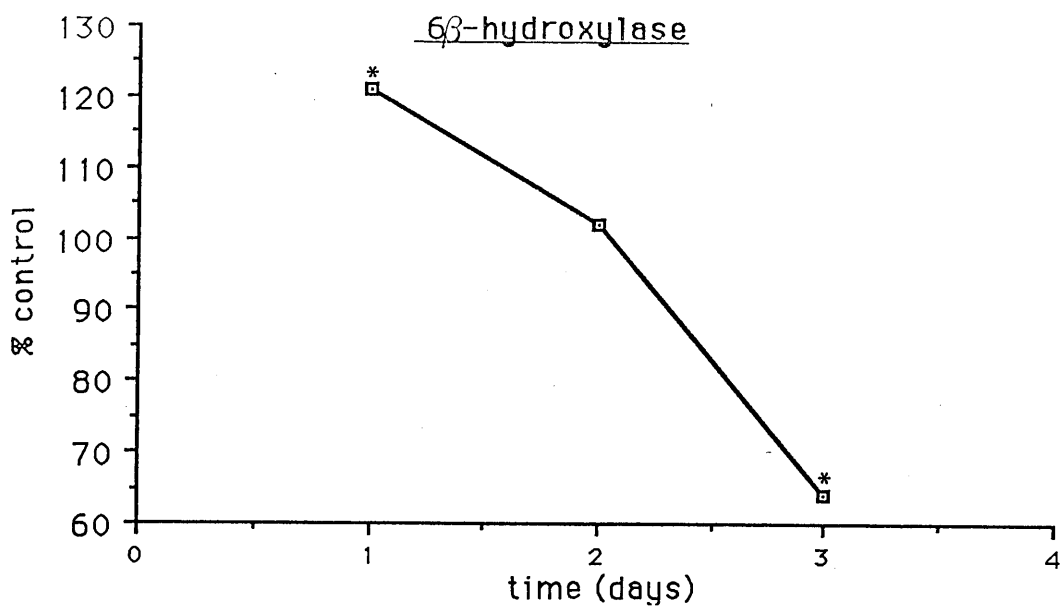


FIGURE 31 : Effects of preincubation with 10^{-6} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 7α -hydroxylase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 22

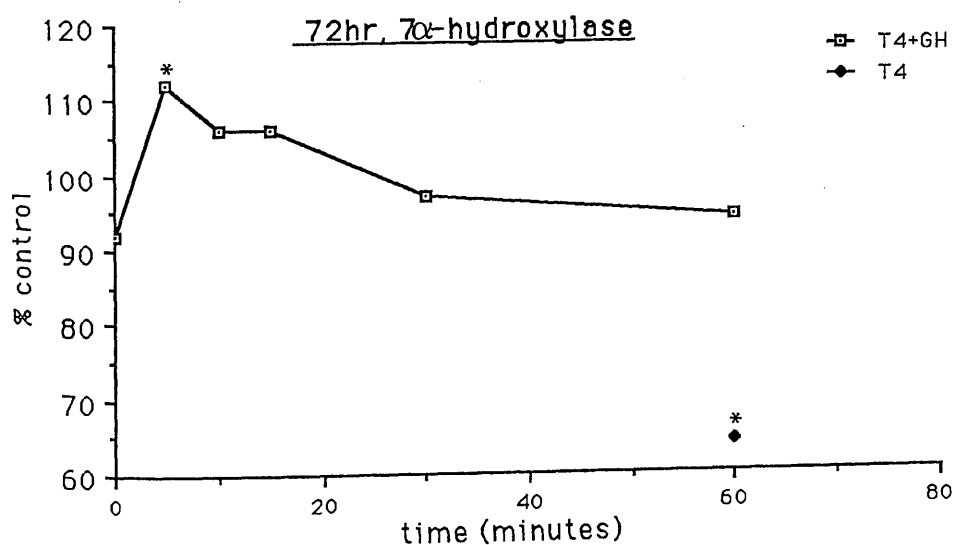
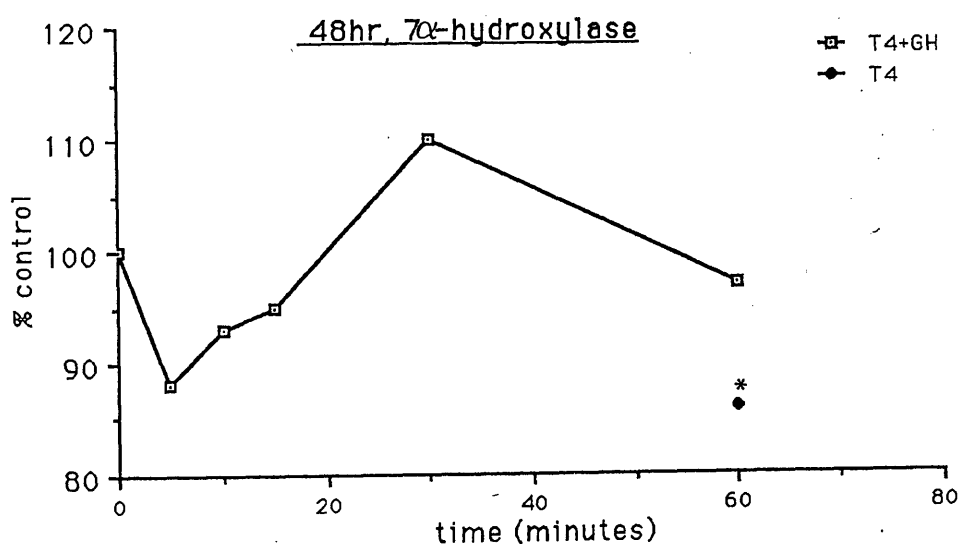
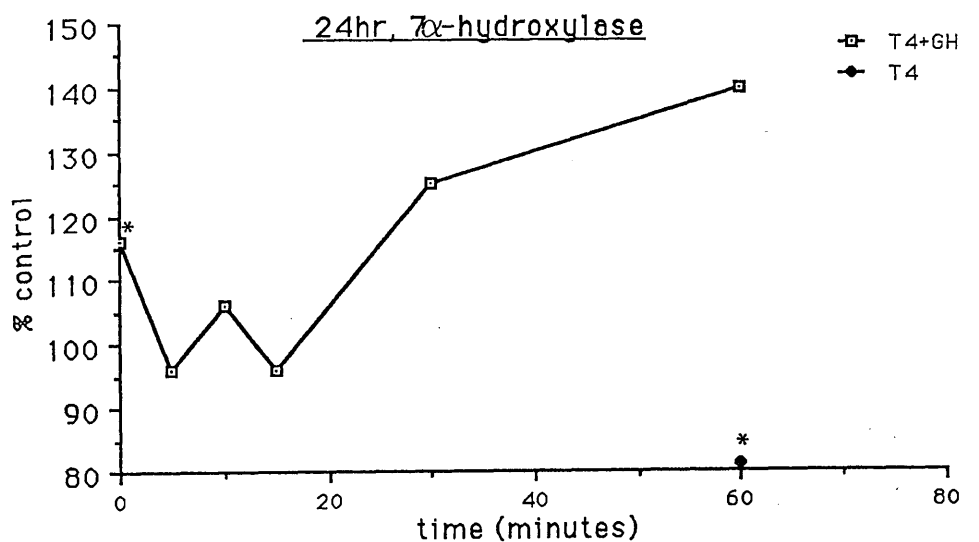


FIGURE 32 : Effects of preincubation with 10^{-6} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 6β -hydroxylase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 22.

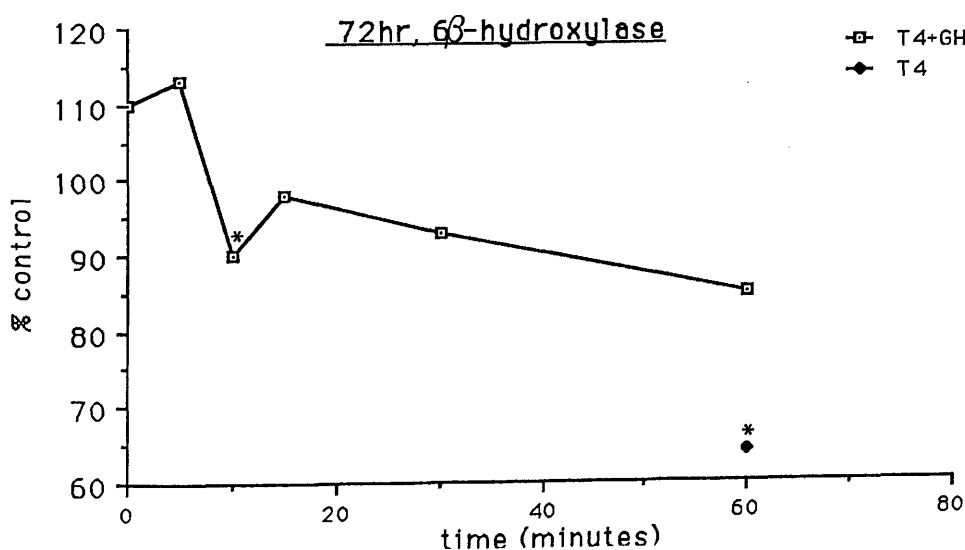
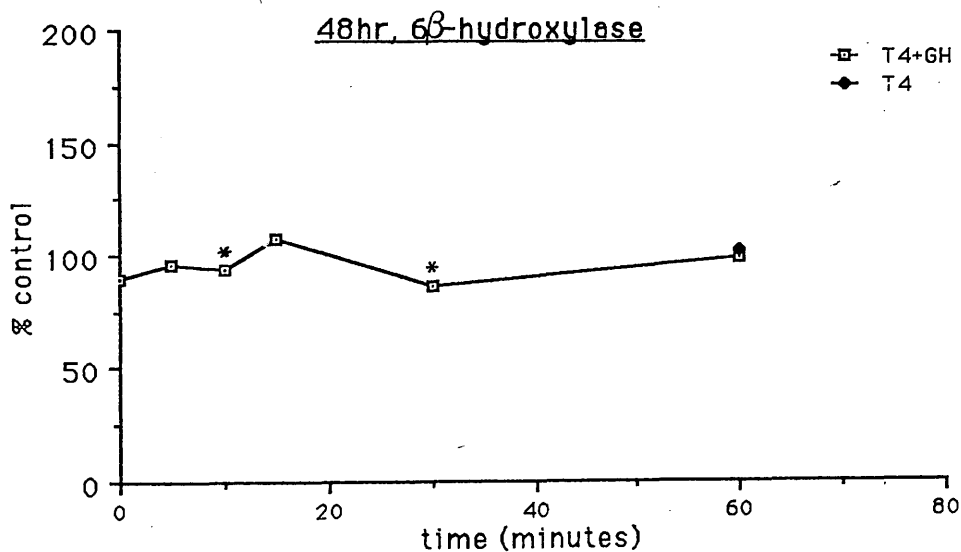
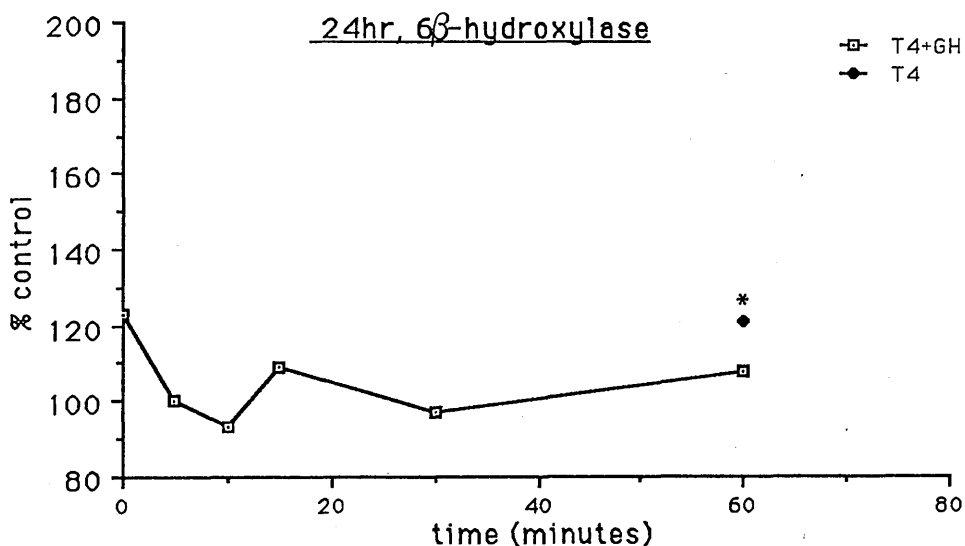


FIGURE 33 : Effects of preincubation with 10^{-6} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 16α -hydroxylase activity. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 22

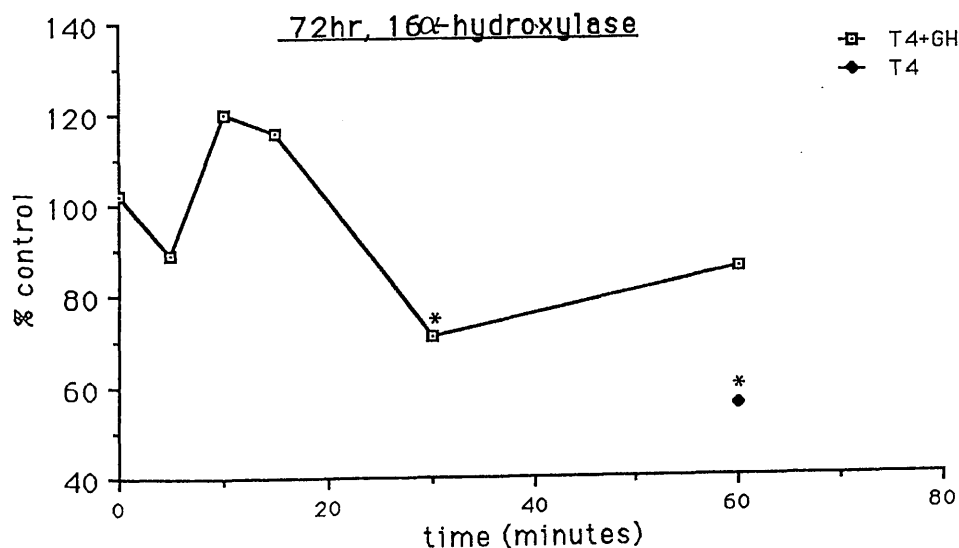
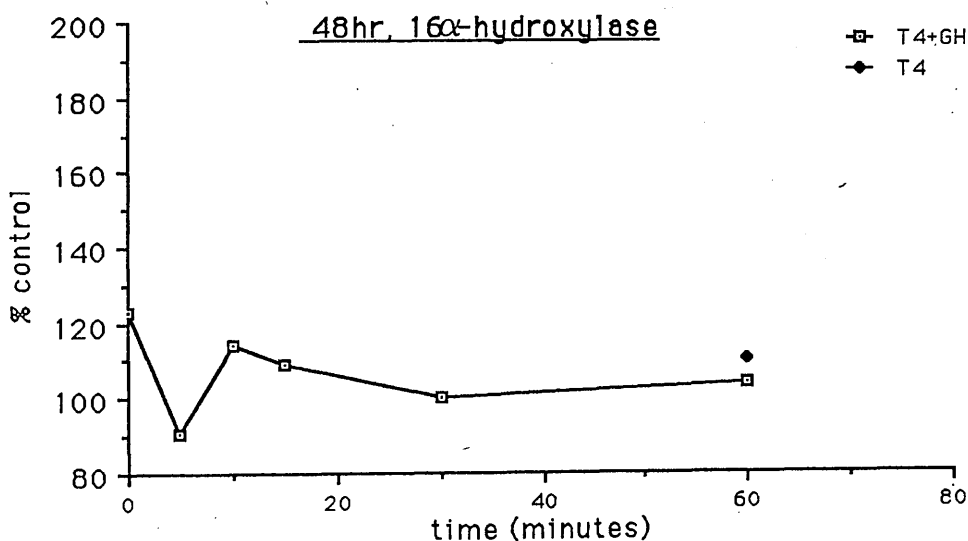
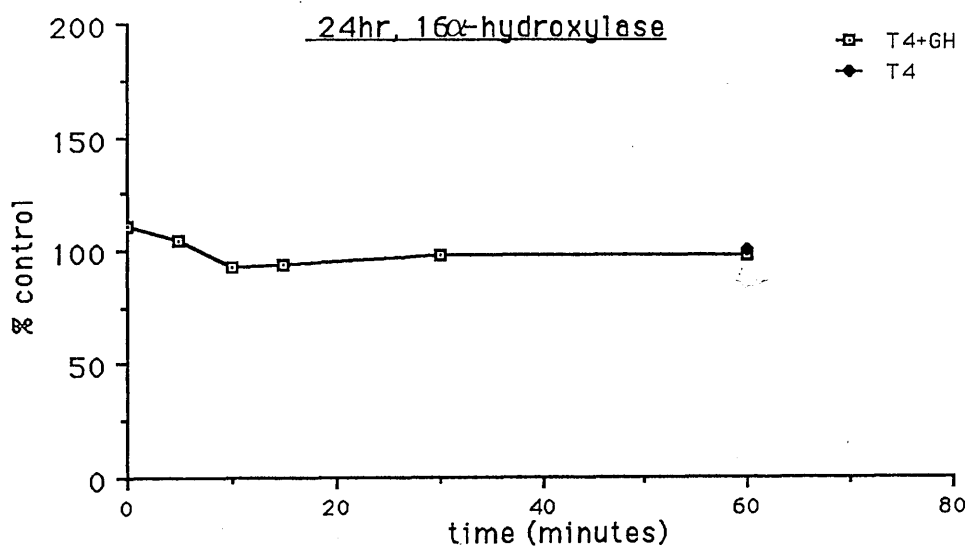


FIGURE 34 : Effects of preincubation with 10^{-6} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 17-oxosteroid oxidoreductase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 22

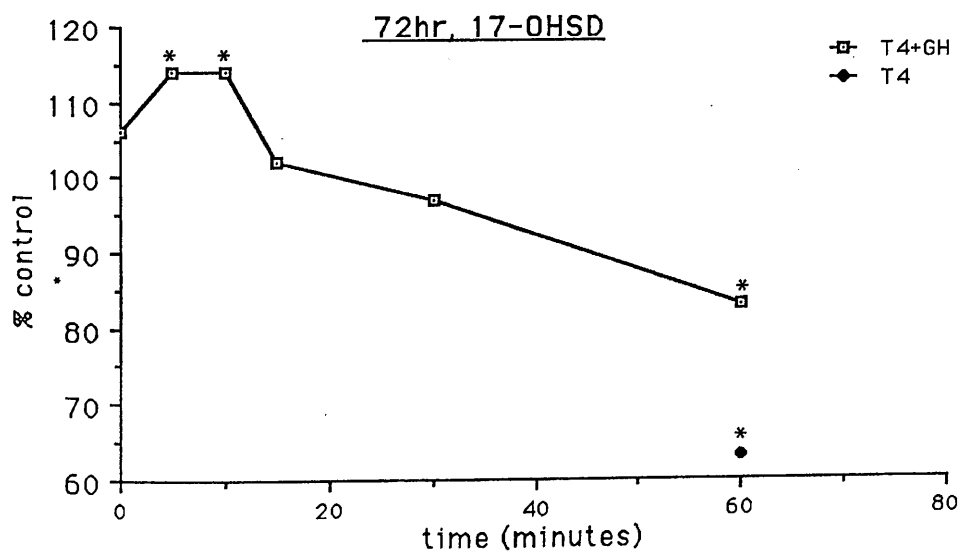
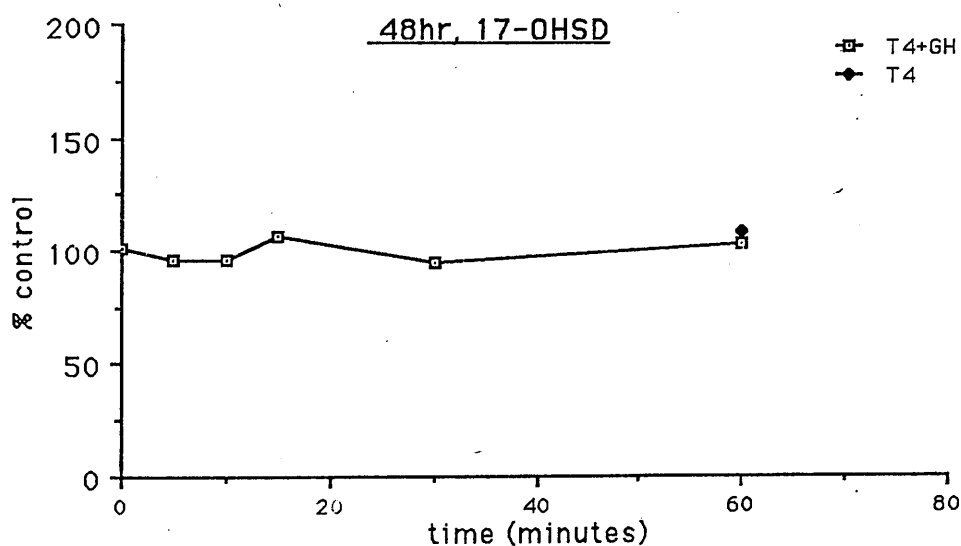
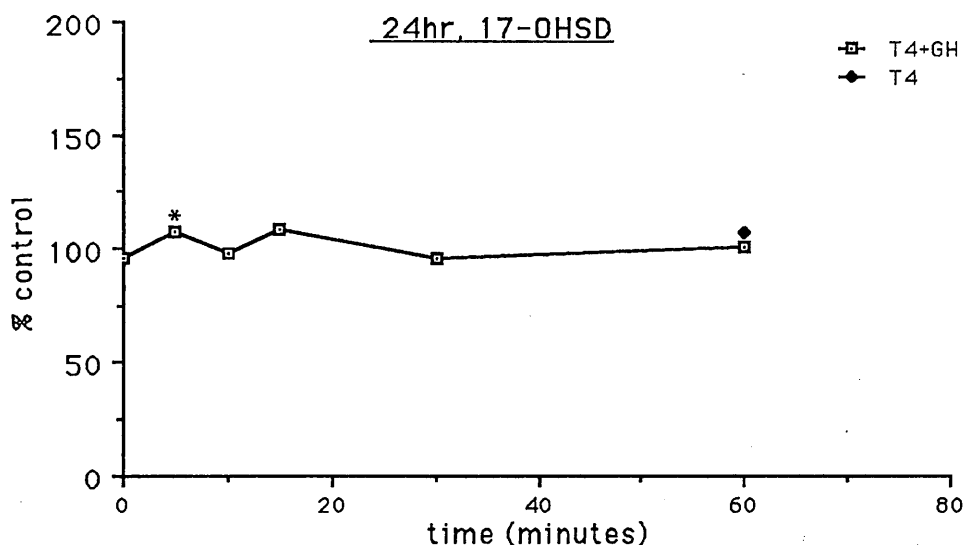


FIGURE 35 : Effects of preincubation with 10^{-6} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 5α -reductase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 22

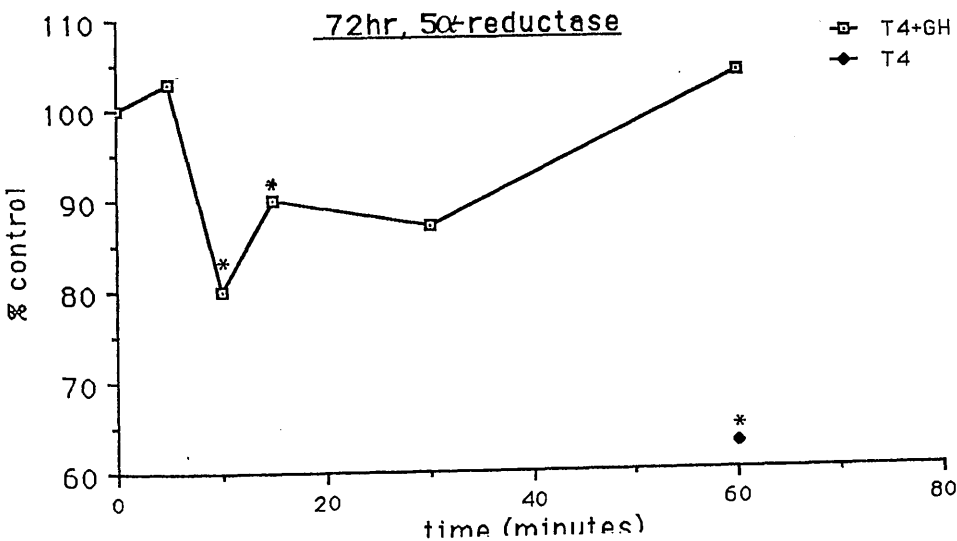
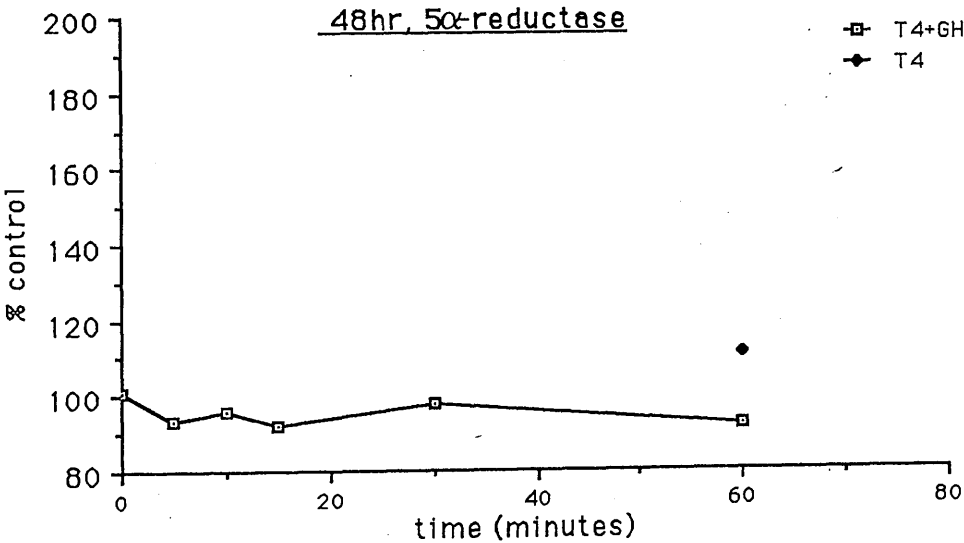
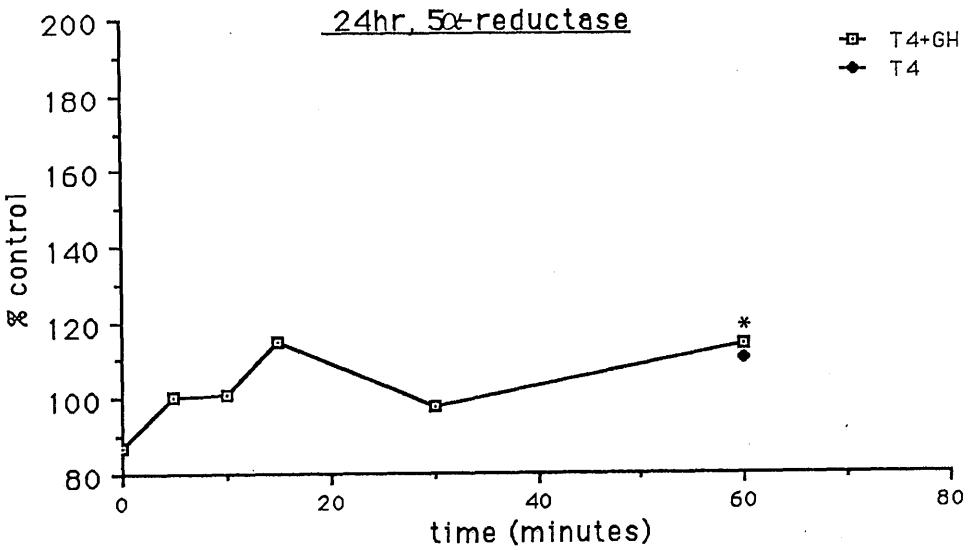
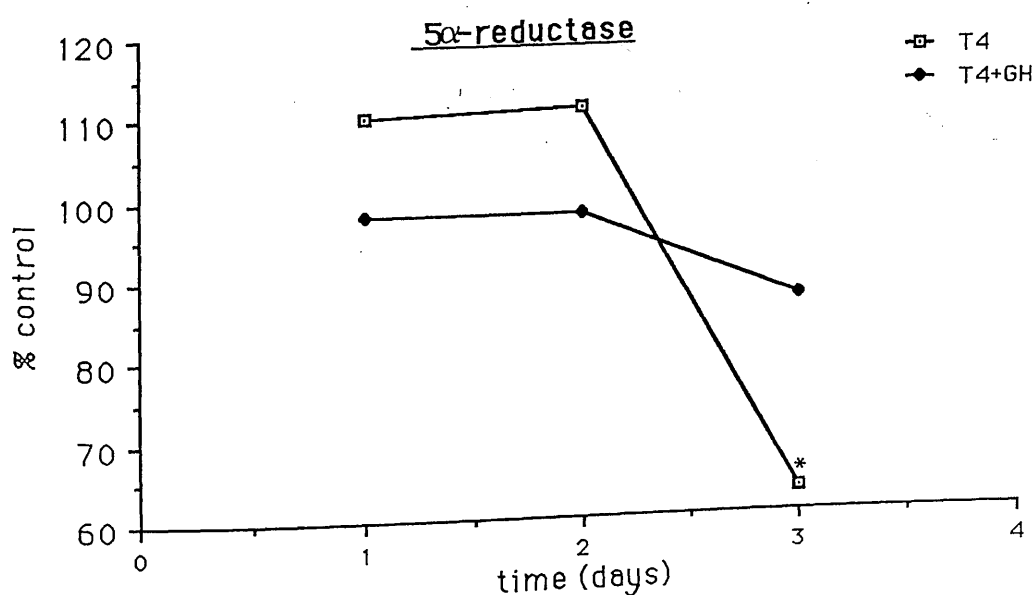
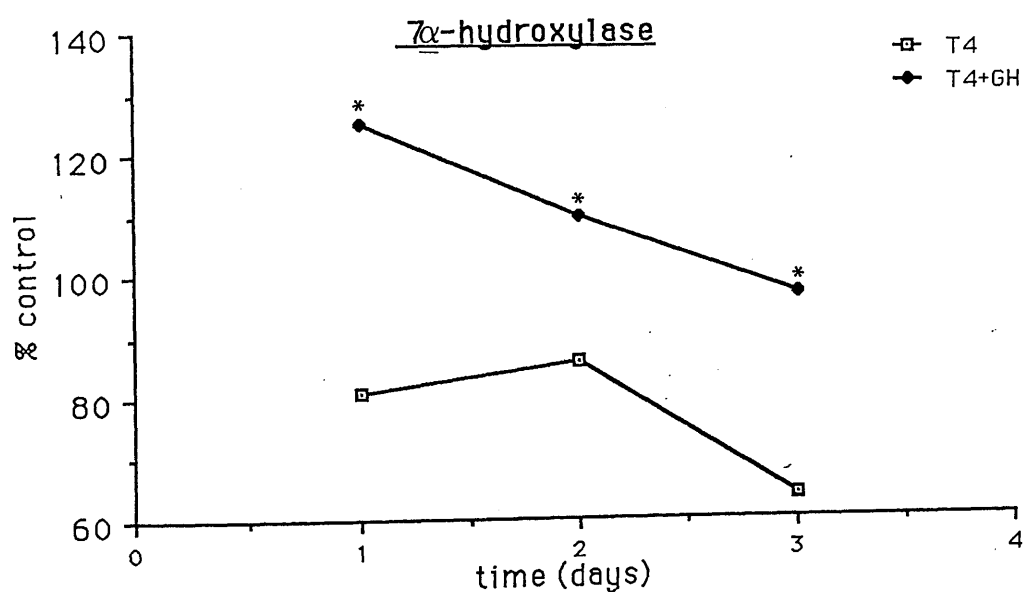


FIGURE 36 : Effects of preincubation with 10^{-6} M THYROXINE over 24, 48 and 72 hours followed by 30 minute incubation with 10^{-9} M GROWTH HORMONE on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 22



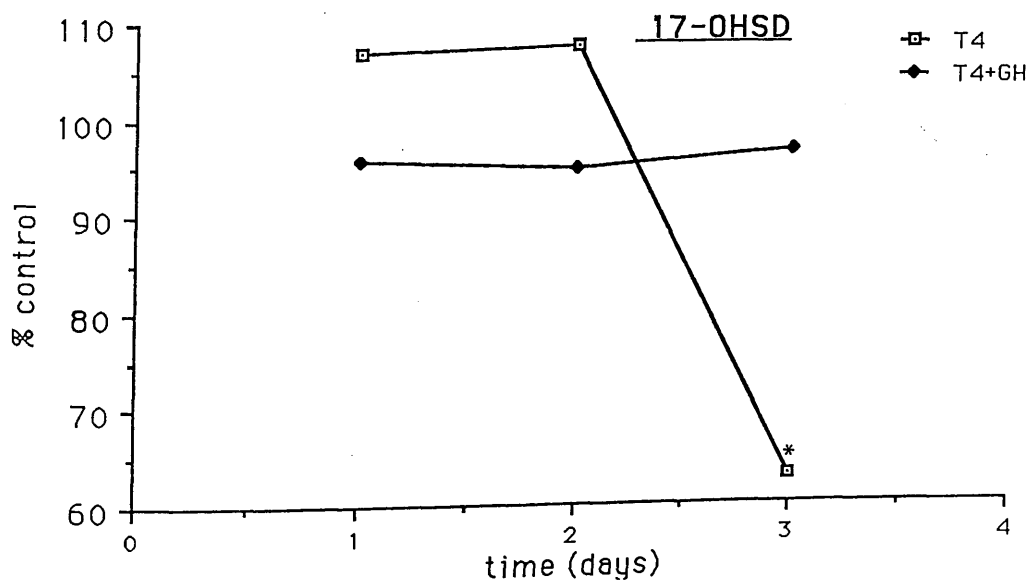
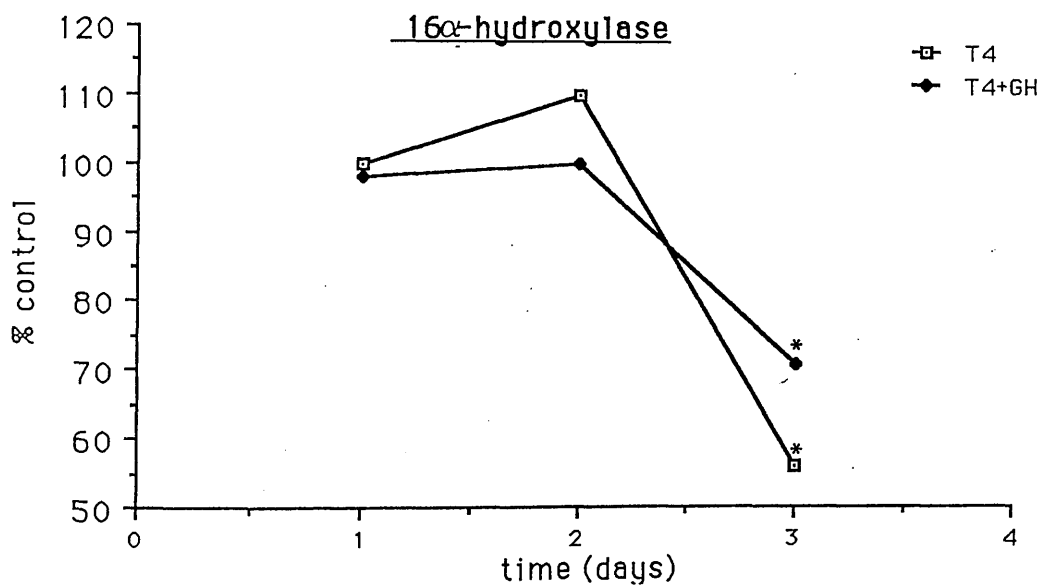
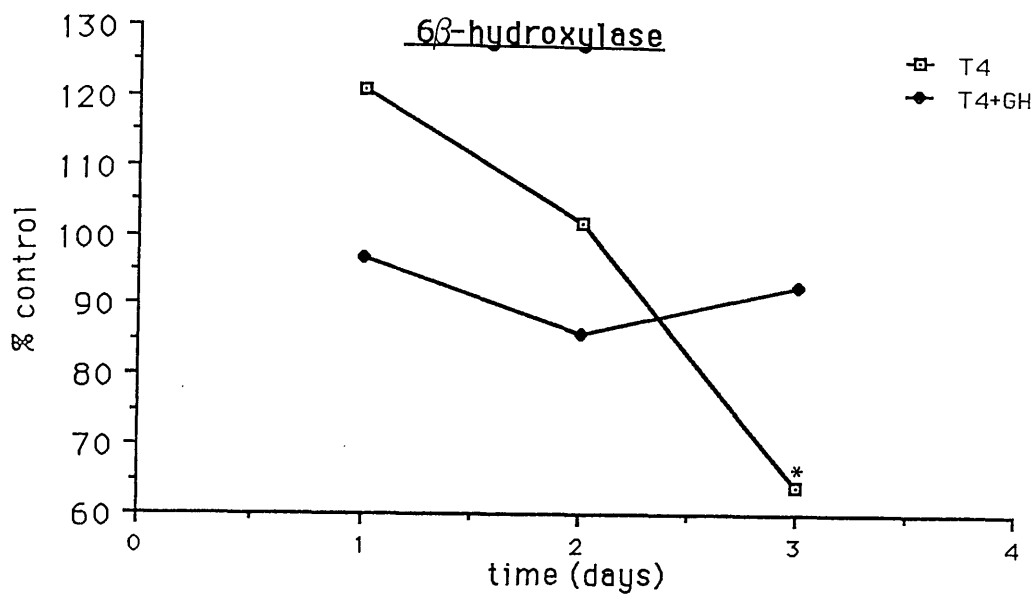


TABLE 22 : Effects of preincubation with 10^{-6} M THYROXINE for 24, 48 and 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes of A) 24h-pretreated, B) 48h-pretreated and C) 72h-pretreated hepatocytes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

A)	Enzyme activities (pmoles/minute/million cells)				
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
24h C	59 \pm 2	53 \pm 3	54 \pm 3	68 \pm 1	100 \pm 3
24h T ₄	48 \pm 1*	64 \pm 3*	54 \pm 3	72 \pm 6	109 \pm 12
control	44 \pm 1	59 \pm 2	45 \pm 4	71 \pm 6	110 \pm 11
24T ₄ +0GH	51 \pm 3*	73 \pm 5	50 \pm 5	68 \pm 4	96 \pm 4*
control	45 \pm 3	62 \pm 6	47 \pm 2	70 \pm 2	99 \pm 7
24T ₄ +5GH	50 \pm 1	62 \pm 1	48 \pm 1	74 \pm 2*	98 \pm 4
control	49 \pm 1	58 \pm 9	51 \pm 4	70 \pm 2	101 \pm 10
24T ₄ +10GH	52 \pm 3	54 \pm 5	47 \pm 1	69 \pm 1	102 \pm 6
control	45 \pm 1	56 \pm 5	50 \pm 1	70 \pm 4	98 \pm 1
24T ₄ +15GH	43 \pm 1	61 \pm 4	47 \pm 5	76 \pm 4	112 \pm 10
control	49 \pm 9	58 \pm 2	54 \pm 6	70 \pm 2	99 \pm 17
24T ₄ +30GH	61 \pm 11	56 \pm 7	53 \pm 2	66 \pm 3	98 \pm 6
control	43 \pm 6	52 \pm 2	51 \pm 1	70 \pm 2	89 \pm 4
24T ₄ +60GH	60 \pm 4	56 \pm 5	50 \pm 1	71 \pm 5	102 \pm 3*

B)	Enzyme activities (pmoles/minute/million cells)				
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
48h C	49 \pm 4	55 \pm 5	49 \pm 2	67 \pm 2	90 \pm 8
48h T ₄	42 \pm 3*	56 \pm 4	54 \pm 2	72 \pm 5	100 \pm 8
control	38 \pm 4	64 \pm 6	44 \pm 8	76 \pm 6	94 \pm 7
48T ₄ +0GH	38 \pm 7	57 \pm 8	54 \pm 4	76 \pm 2	95 \pm 8
control	41 \pm 3	68 \pm 4	45 \pm 7	78 \pm 3	102 \pm 7
48T ₄ +5GH	36 \pm 5	65 \pm 4	41 \pm 3	74 \pm 4	96 \pm 4
control	44 \pm 2	62 \pm 2	42 \pm 2	80 \pm 8	99 \pm 5
48T ₄ +10GH	41 \pm 3	58 \pm 2*	48 \pm 6	77 \pm 6	95 \pm 6
control	38 \pm 3	58 \pm 4	44 \pm 4	78 \pm 3	100 \pm 6
48T ₄ +15GH	36 \pm 5	62 \pm 5	48 \pm 5	82 \pm 2	92 \pm 8
control	39 \pm 9	70 \pm 2	42 \pm 6	78 \pm 2	96 \pm 4
48T ₄ +30GH	43 \pm 5	60 \pm 6*	42 \pm 2	74 \pm 4	94 \pm 8
control	44 \pm 4	55 \pm 8	47 \pm 6	75 \pm 4	99 \pm 7
48T ₄ +60GH	42 \pm 4	54 \pm 2	49 \pm 6	78 \pm 5	92 \pm 3

C)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
72h C		61 \pm 2	74 \pm 4	73 \pm 3	110 \pm 1	142 \pm 4
72h T ₄		39 \pm 2*	47 \pm 3*	41 \pm 10*	69 \pm 2*	89 \pm 3*
control		38 \pm 6	48 \pm 7	41 \pm 5	63 \pm 9	84 \pm 9
72T ₄ +0GH		35 \pm 3	53 \pm 6	42 \pm 9	65 \pm 2	84 \pm 4
control		32 \pm 2	46 \pm 2	44 \pm 8	60 \pm 4	82 \pm 8
72T ₄ +5GH		36 \pm 2*	52 \pm 9	39 \pm 7	68 \pm 2*	84 \pm 5
control		33 \pm 3	59 \pm 5	39 \pm 3	60 \pm 2	95 \pm 4
72T ₄ +10GH		35 \pm 4	53 \pm 1*	47 \pm 4	68 \pm 4*	84 \pm 5*
control		34 \pm 7	52 \pm 7	38 \pm 4	61 \pm 4	88 \pm 4
72T ₄ +15GH		36 \pm 7	51 \pm 2	44 \pm 6	64 \pm 5	79 \pm 6*
control		35 \pm 3	55 \pm 2	45 \pm 1	70 \pm 6	62 \pm 10
72T ₄ +30GH		34 \pm 3	61 \pm 6	32 \pm 4*	68 \pm 4	80 \pm 6
control		35 \pm 2	59 \pm 1	42 \pm 2	68 \pm 6	83 \pm 2
72T ₄ +60GH		33 \pm 6	50 \pm 10	36 \pm 8	56 \pm 5*	86 \pm 6

Results are expressed as mean \pm s.d. (N=3); *=P<0.05 as compared to relevant control values (C=control).

Results given in Figures 30 to 35 are summarised in Figure 36. Growth hormone modulated the effects of thyroxine restoring all enzyme activities to basal levels.

3.4.5 PREINCUBATION OF HEPATOCYTES WITH THYROXINE
(10^{-8} M) FOR 24, 48 AND 72 HOURS FOLLOWED BY INCUBATION
WITH 10^{-9} M GROWTH HORMONE OVER 0 TO 60 MINUTES

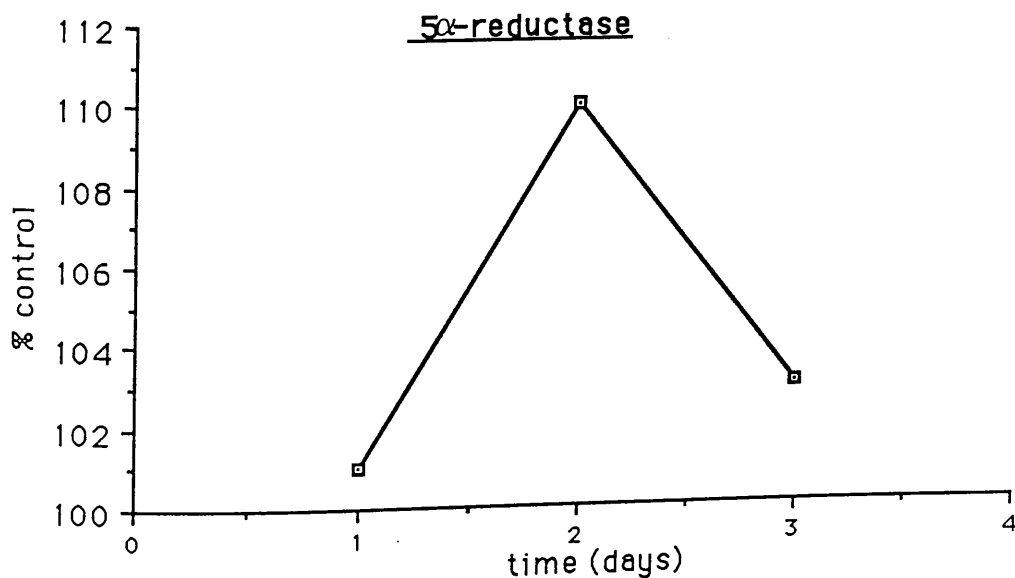
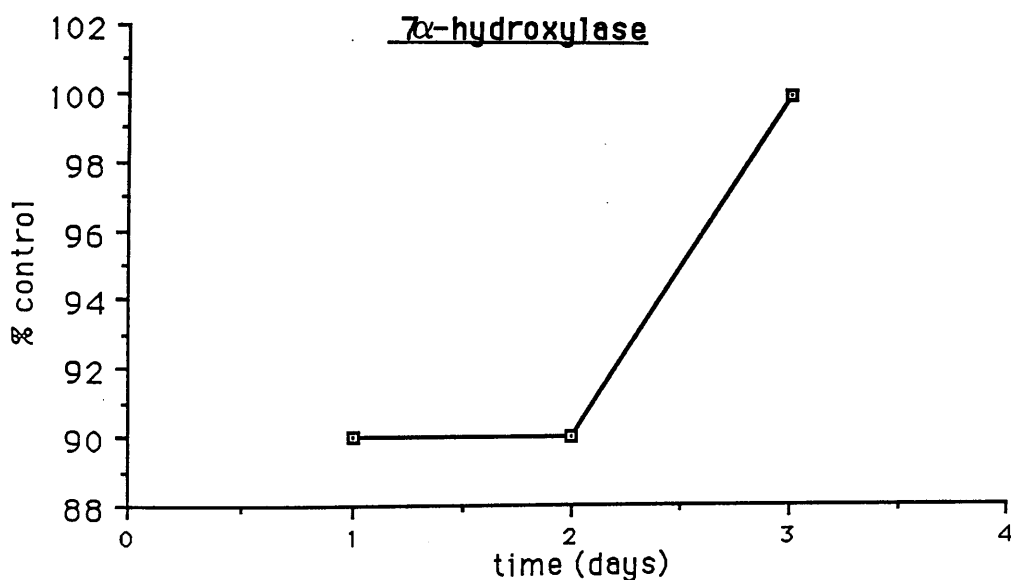
Incubation of hepatocytes with 10^{-8} M thyroxine (Table 23) resulted in no significant changes in enzyme activity (Figure 37) except for significant differences in 6β -hydroxylase activity (Figure 37C) and 16α -hydroxylase activity (Figure 37D).

Further incubation of thyroxine-treated hepatocytes with 10^{-9} M growth hormone over 0 to 60 minutes resulted in no significant differences in 7α -hydroxylase activity (Figure 38). 6β -hydroxylase activity (Figure 39) was significantly increased back to basal levels with 72h thyroxine-pretreated hepatocytes upon incubation with growth hormone, although this effect was not significant with respect to relevant control incubations. No changes were exhibited upon further treatment of 24- and 48h pretreated hepatocytes with growth hormone.

No significant changes were observed in 16α -hydroxylase, 17α -oxosteroid oxidoreductase and 5α -reductase activities (Figure 40, 41 and 42 respectively) upon further treatment with growth hormone as compared to both the thyroxine response and relevant control values.

The results from Figures 37 to 42 are summarised in

FIGURE 37 : Effects of preincubation with 10^{-8} M THYROXINE over 24, 48 and 72 hours on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 23



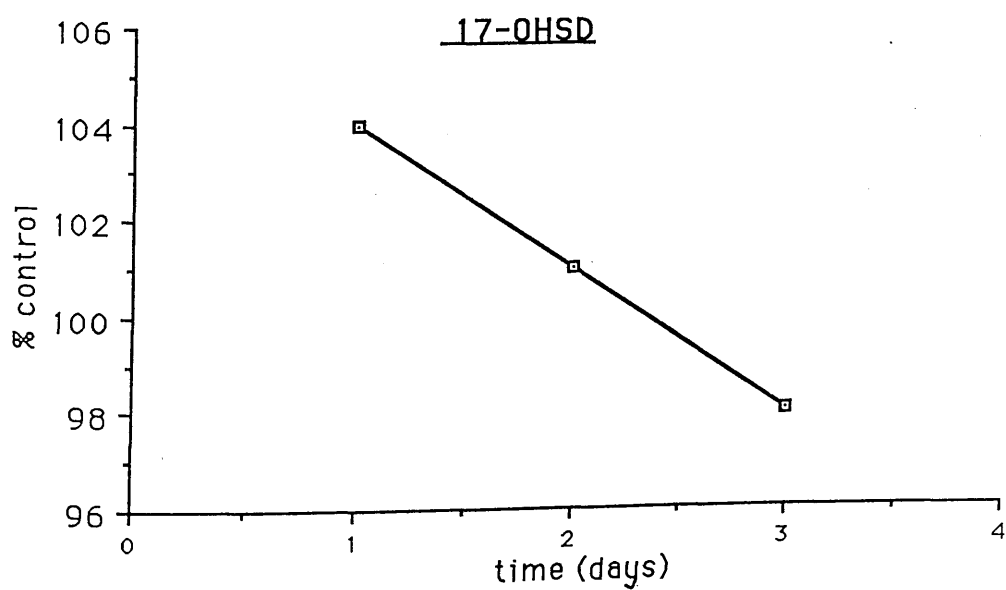
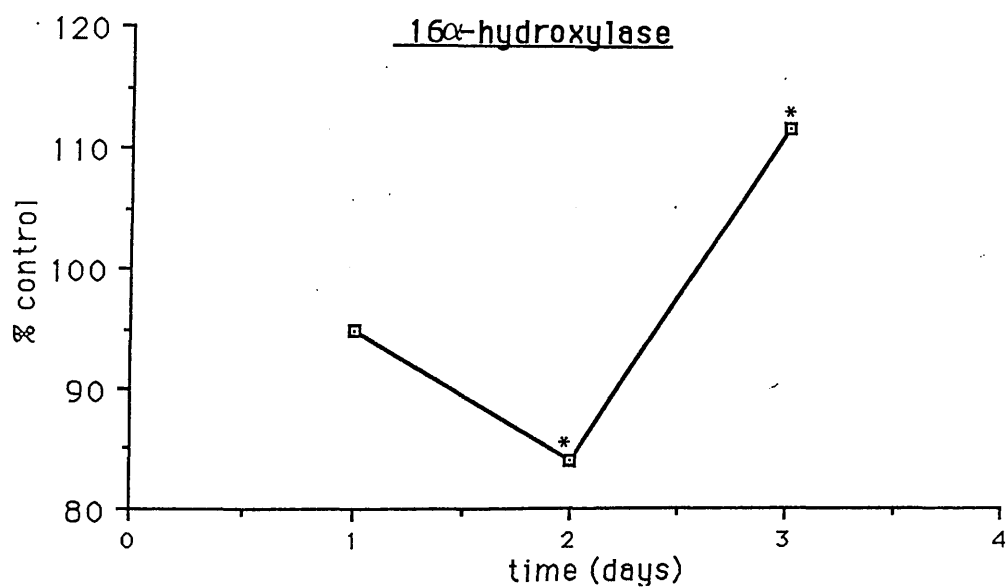
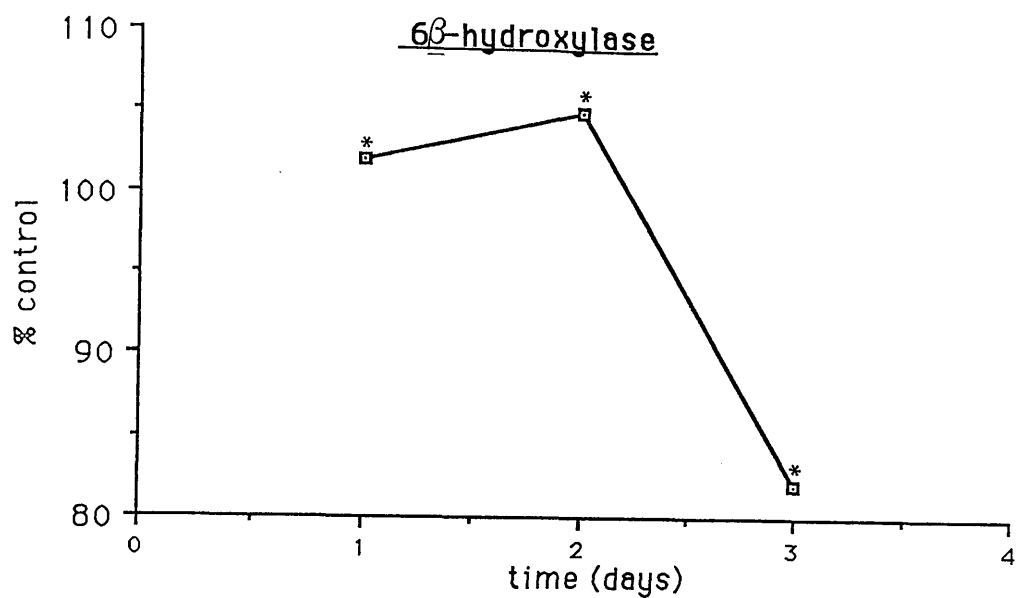


FIGURE 38 : Effects of preincubation with 10^{-8} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 7α -hydroxylase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 23

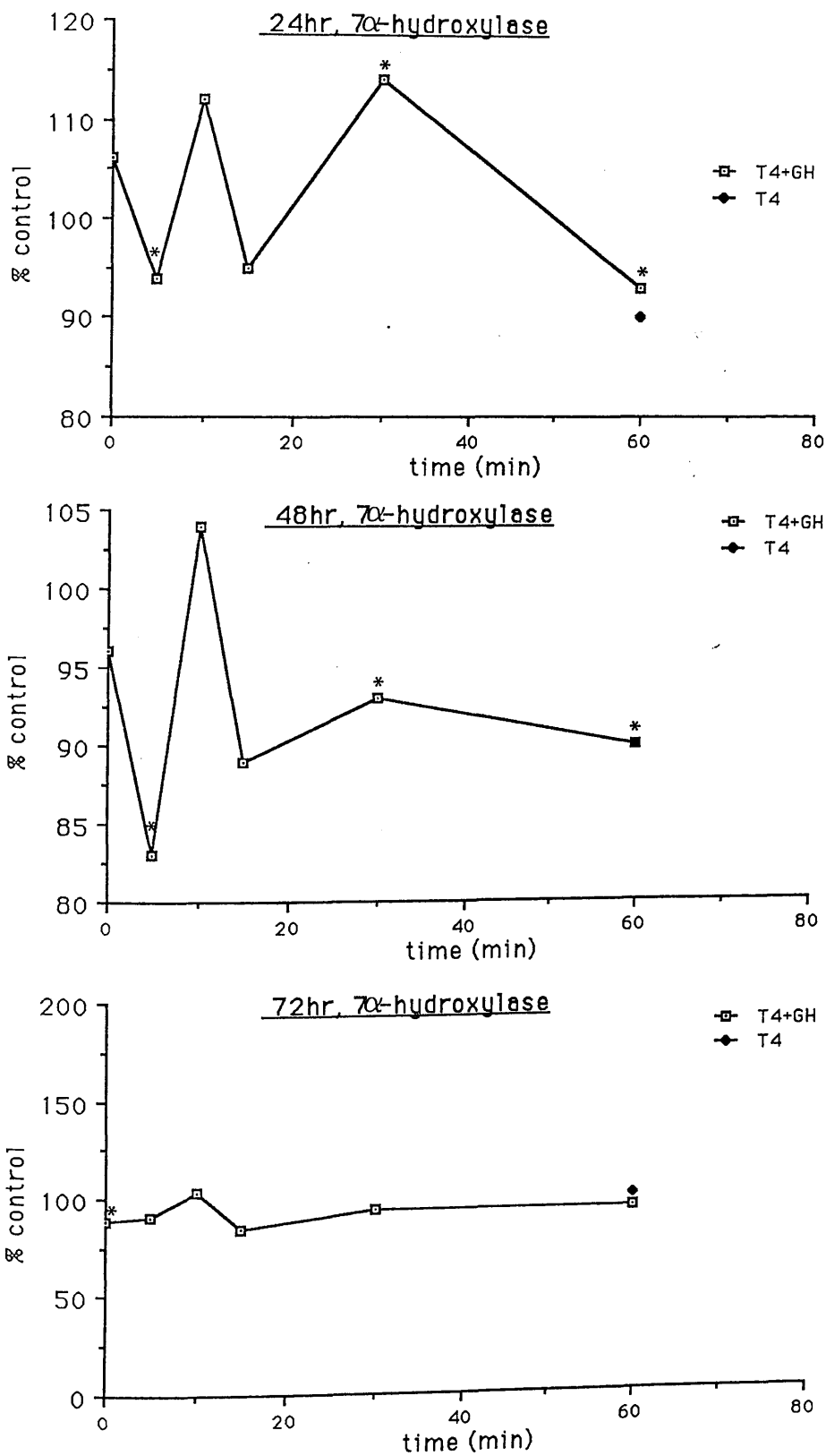


FIGURE 39 : Effects of preincubation with 10^{-8} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 6β -hydroxylase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 23

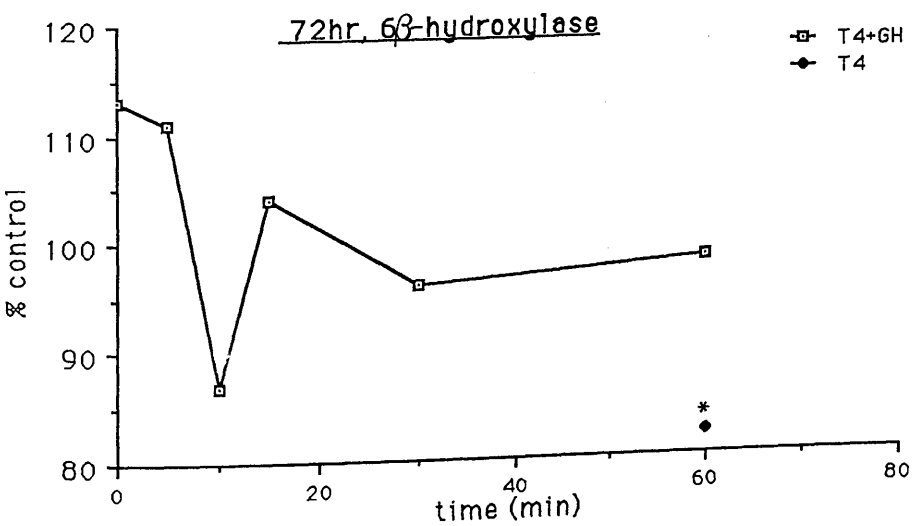
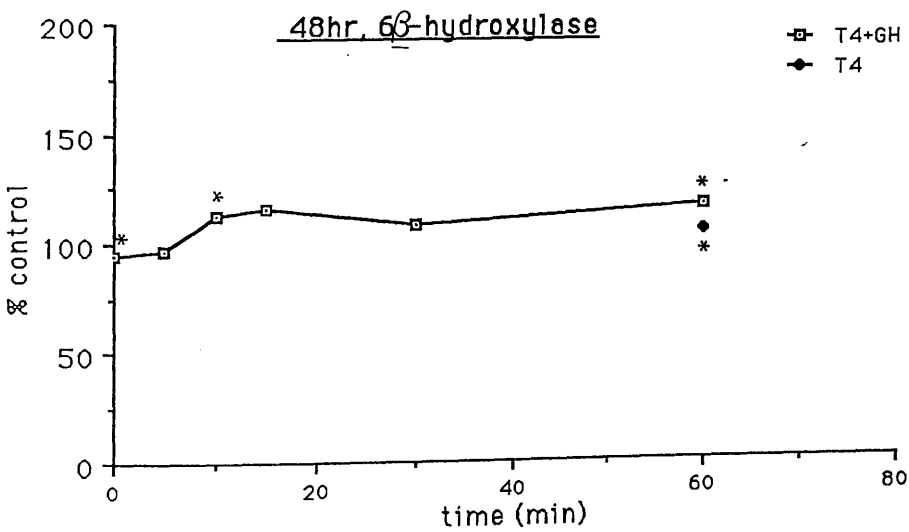
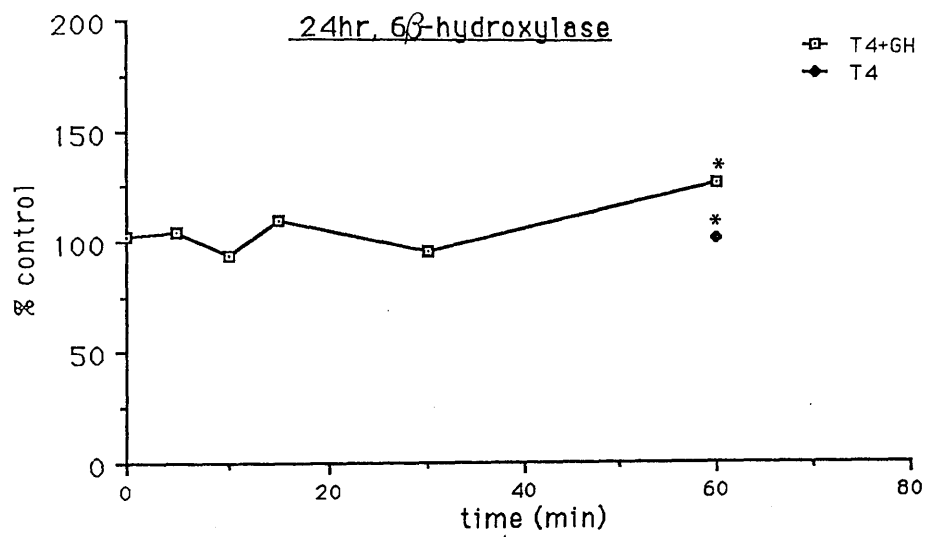


FIGURE 40 : Effects of preincubation with 10^{-8} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 16α -hydroxylase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 23

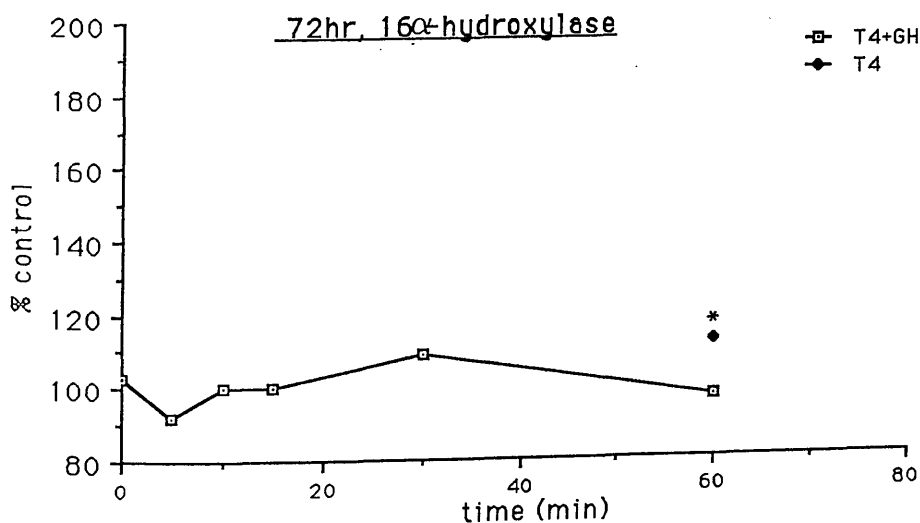
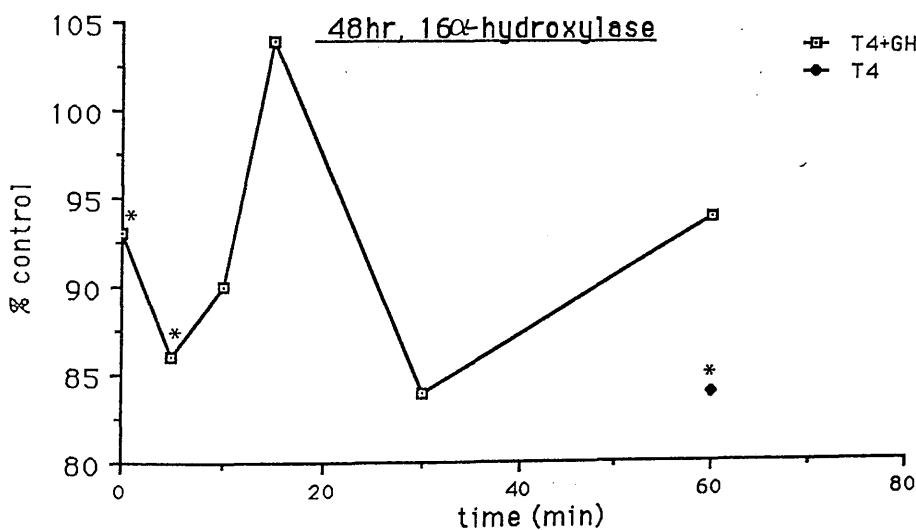
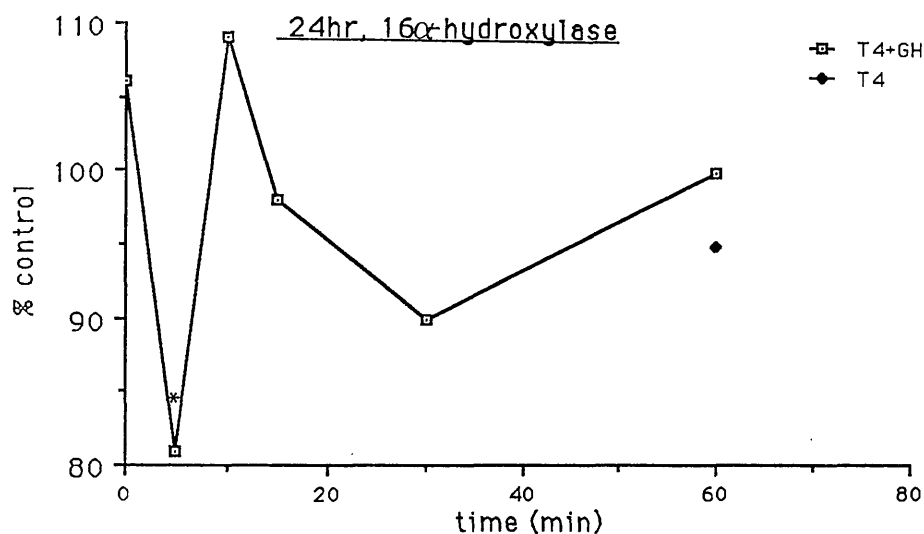


FIGURE 41 : Effects of preincubation with 10^{-8} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 17-oxosteroid oxidoreductase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 23

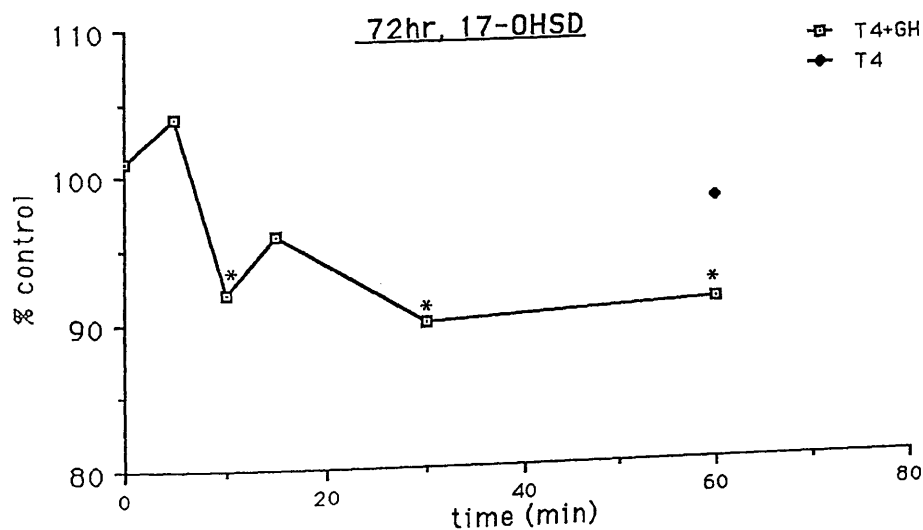
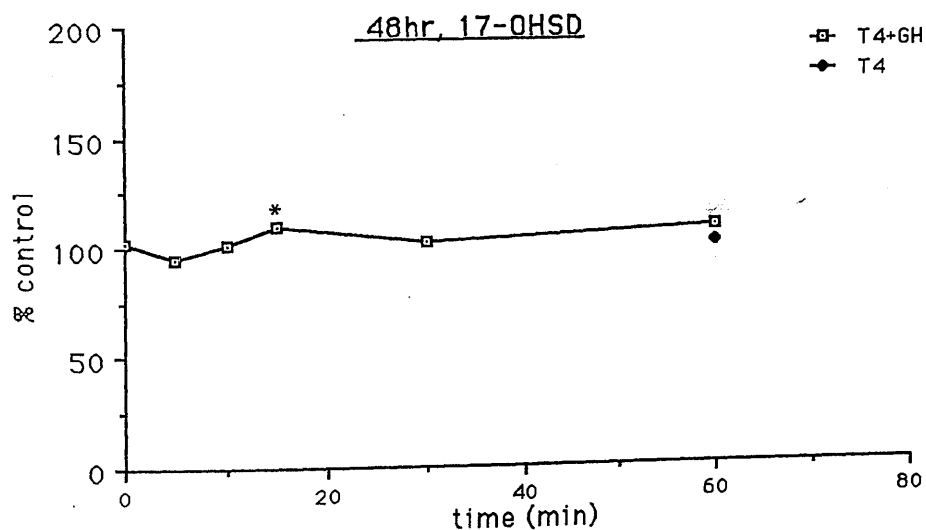
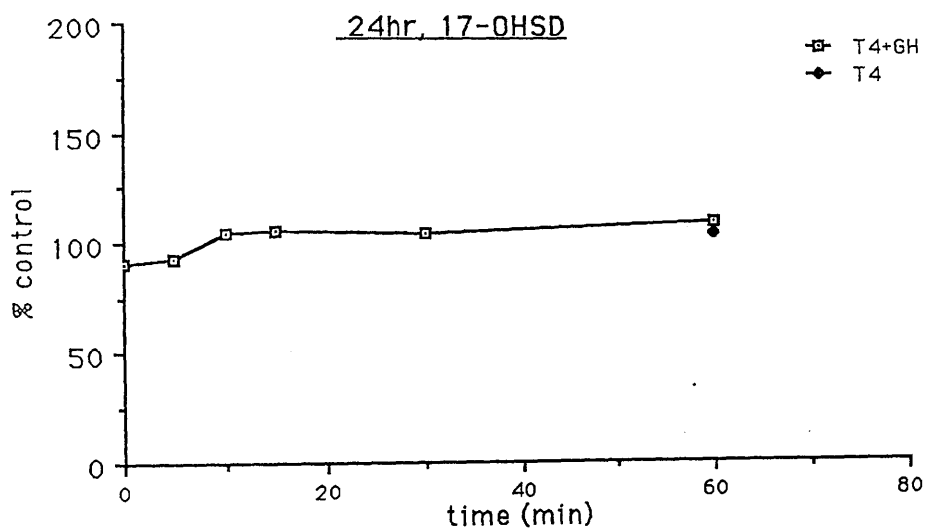


FIGURE 42 : Effects of preincubation with 10^{-8} M THYROXINE for A) 24 hours, B) 48 hours and C) 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes on 5 α -reductase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 23

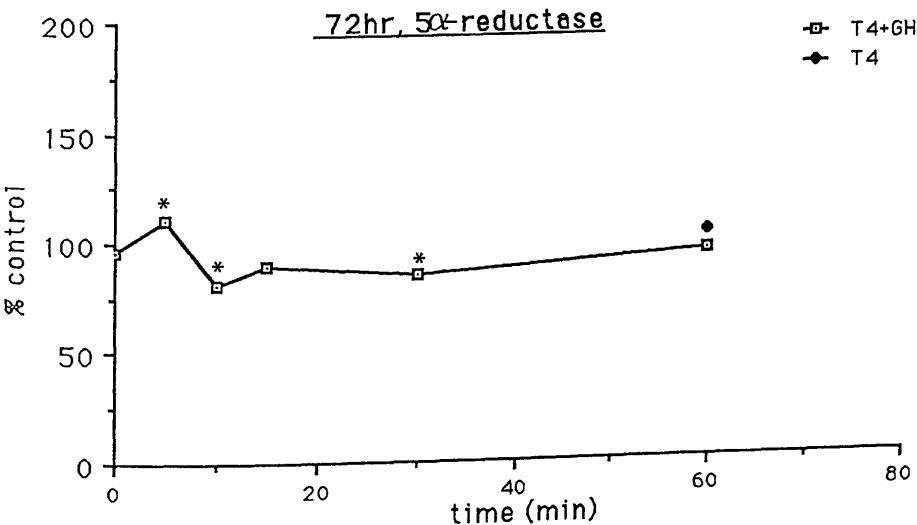
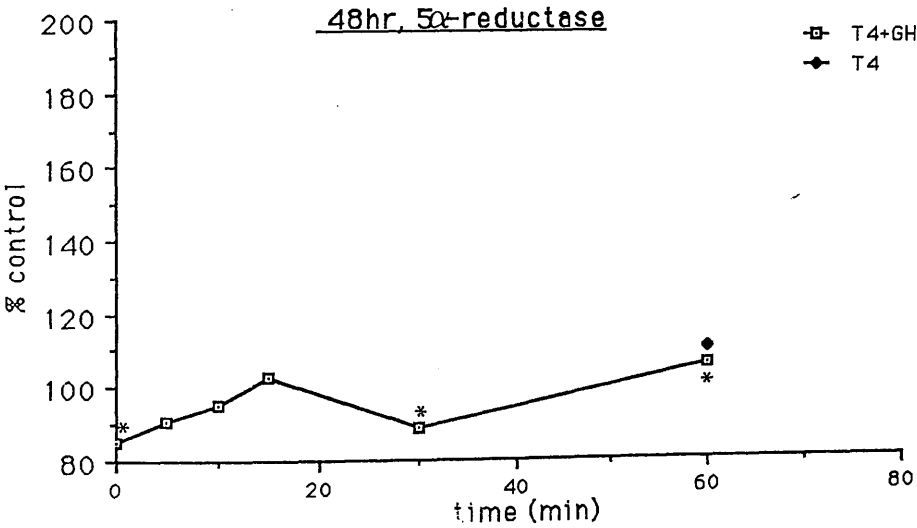
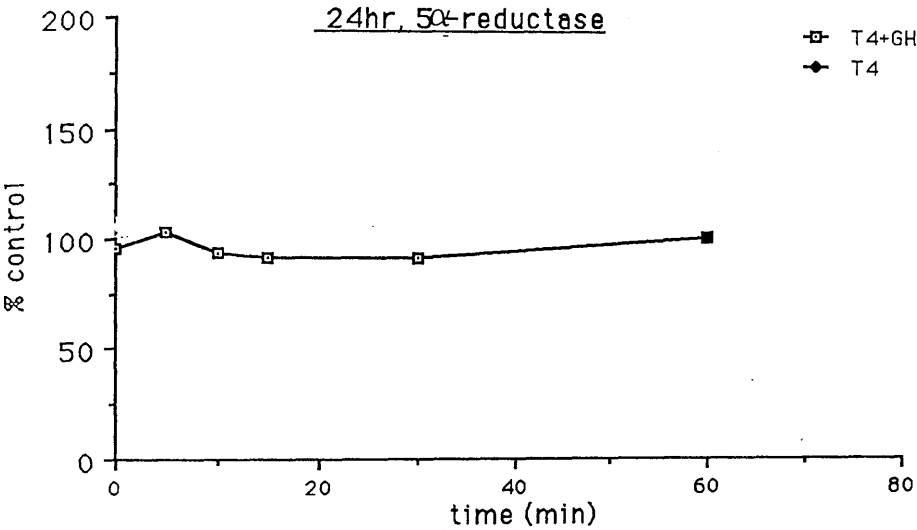
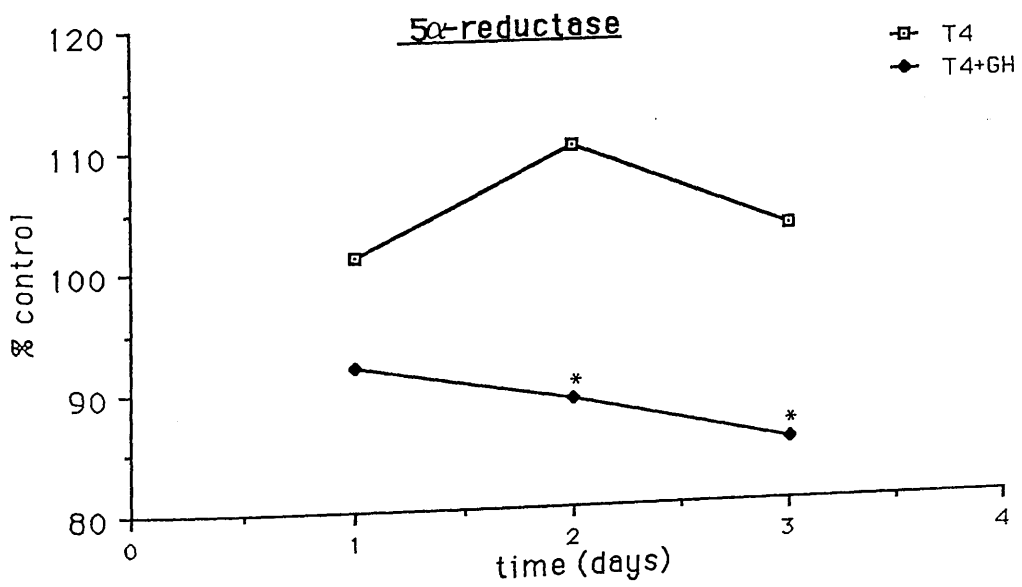
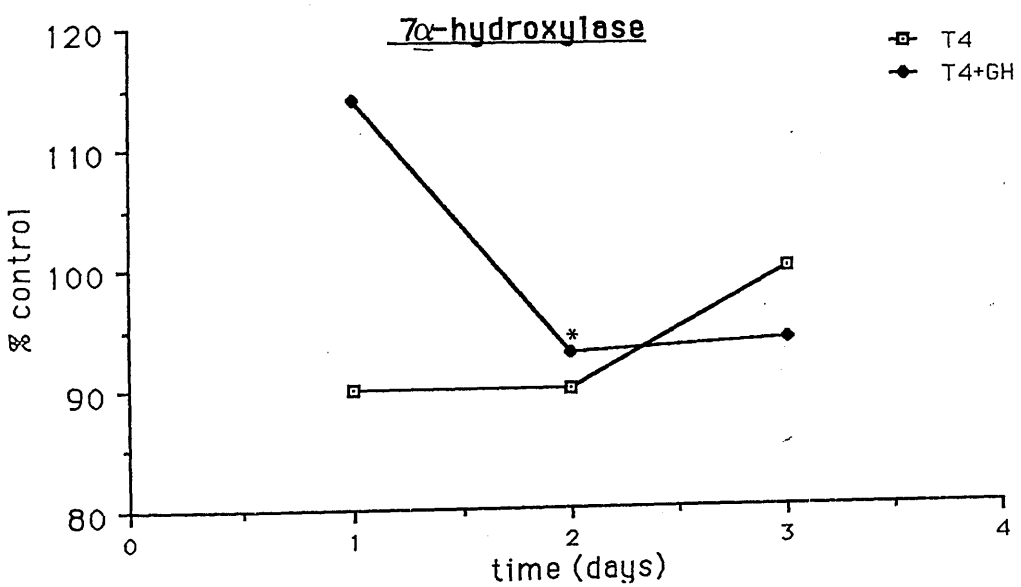


FIGURE 43 : Effects of preincubation with 10^{-8} M THYROXINE over 24, 48 and 72 hours followed by 30 minute incubation with 10^{-9} M GROWTH HORMONE on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 23



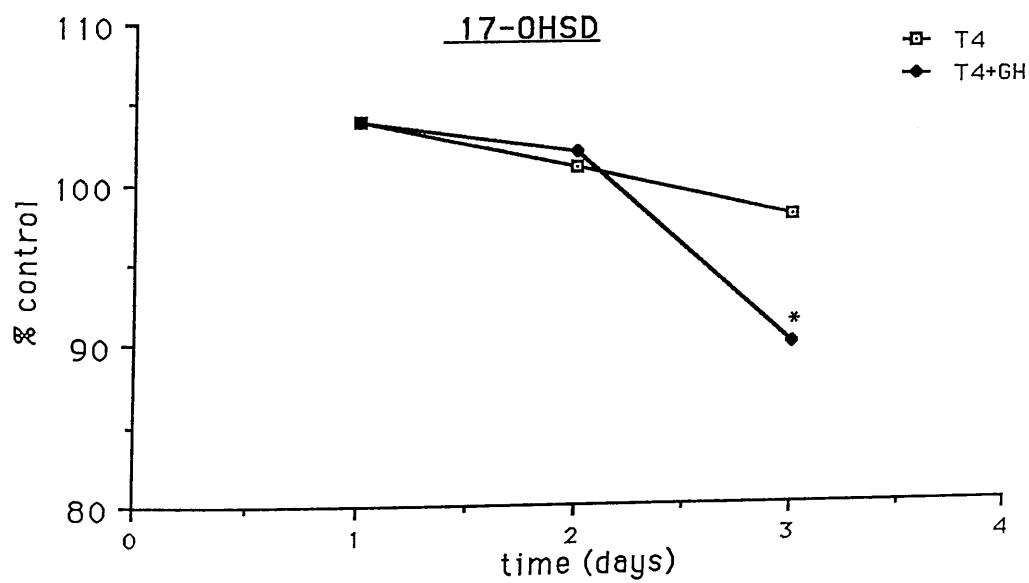
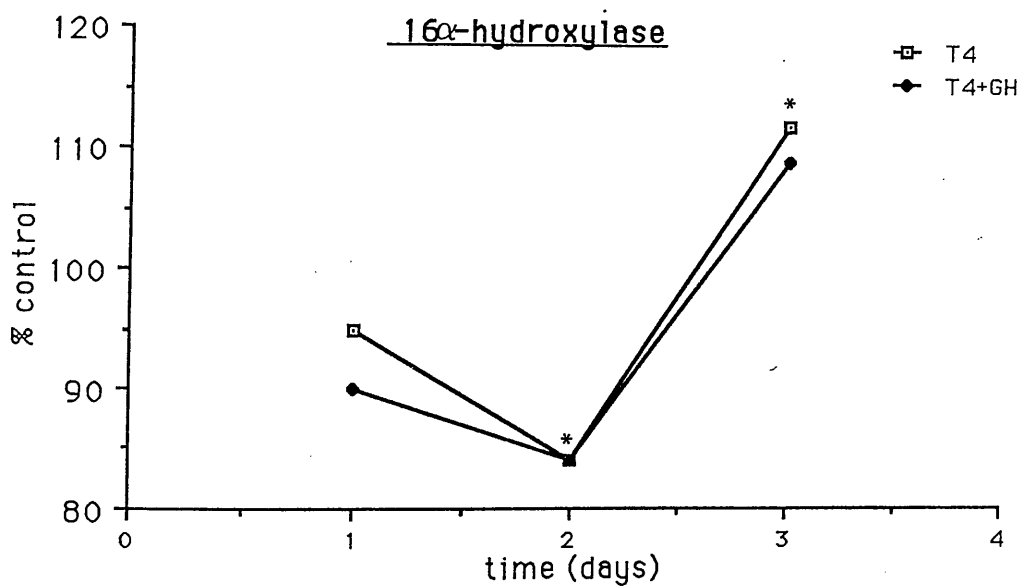
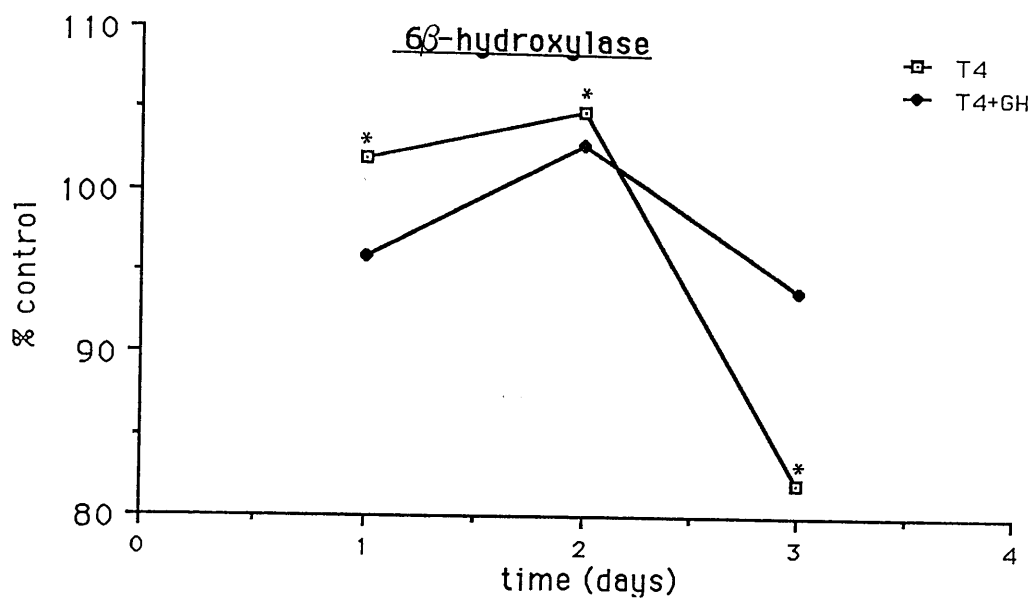


TABLE 23 : Effects of preincubation with 10^{-8} M THYROXINE for 24, 48 and 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE over 0 to 60 minutes of A) 24h-pretreated, B) 48h-pretreated and C) 72h-pretreated hepatocytes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

A)	Enzyme activities (pmoles/minute/million cells)				
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red ^d
24h C	38 \pm 3	43 \pm 2	39 \pm 4	110 \pm 4	88 \pm 1
24h T ₄	38 \pm 5	47 \pm 1*	37 \pm 5	115 \pm 5	88 \pm 4
control	34 \pm 1	45 \pm 2	33 \pm 1	117 \pm 9	76 \pm 1
24T ₄ +0GH	36 \pm 2	46 \pm 2	35 \pm 4	107 \pm 6	78 \pm 3
control	34 \pm 3	47 \pm 1	42 \pm 4	121 \pm 9	76 \pm 8
24T ₄ +5GH	32 \pm 3	49 \pm 2	34 \pm 3*	113 \pm 6	80 \pm 9
control	34 \pm 1	52 \pm 1	34 \pm 2	110 \pm 3	86 \pm 6
24T ₄ +10GH	38 \pm 4	49 \pm 6	37 \pm 3	114 \pm 7	78 \pm 5
control	38 \pm 3	46 \pm 2	40 \pm 5	119 \pm 6	88 \pm 8
24T ₄ +15GH	36 \pm 6	50 \pm 9	39 \pm 6	125 \pm 13	76 \pm 2
control	36 \pm 3	47 \pm 2	41 \pm 4	118 \pm 5	88 \pm 7
24T ₄ +30GH	41 \pm 5	45 \pm 3	37 \pm 2	123 \pm 12	76 \pm 4
control	41 \pm 8	45 \pm 3	38 \pm 5	121 \pm 7	86 \pm 8
24T ₄ +60GH	38 \pm 2	57 \pm 6*	38 \pm 2	132 \pm 9	86 \pm 5

B)	Enzyme activities (pmoles/minute/million cells)				
TIME(min)	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
48h C	31 \pm 3	40 \pm 1	31 \pm 2	92 \pm 2	66 \pm 6
48h T ₄	28 \pm 4	43 \pm 1*	26 \pm 1*	92 \pm 2	65 \pm 5
control	26 \pm 3	39 \pm 1	30 \pm 1	91 \pm 4	70 \pm 4
48T ₄ +0GH	25 \pm 2	37 \pm 1*	28 \pm 1*	92 \pm 6	59 \pm 2*
control	29 \pm 3	38 \pm 2	28 \pm 1	97 \pm 6	68 \pm 4
48T ₄ +5GH	24 \pm 2*	37 \pm 1	24 \pm 2*	92 \pm 6	62 \pm 6
control	25 \pm 2	39 \pm 1	29 \pm 1	93 \pm 5	66 \pm 5
48T ₄ +10GH	26 \pm 2	44 \pm 3*	26 \pm 4	94 \pm 9	62 \pm 6
control	28 \pm 2	37 \pm 4	28 \pm 2	89 \pm 3	63 \pm 1
48T ₄ +15GH	25 \pm 3	43 \pm 3	29 \pm 3	97 \pm 2*	65 \pm 5
control	27 \pm 1	37 \pm 5	31 \pm 4	89 \pm 2	68 \pm 3
48T ₄ +30GH	25 \pm 1*	40 \pm 2	26 \pm 3	91 \pm 6	61 \pm 4*
control	25 \pm 2	39 \pm 3	31 \pm 3	105 \pm 5	63 \pm 2
48T ₄ +60GH	32 \pm 2*	46 \pm 5*	29 \pm 2	112 \pm 4	67 \pm 1*

C)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
72h C		38 \pm 2	51 \pm 2	41 \pm 1	114 \pm 7	84 \pm 5
72h T ₄		38 \pm 2	42 \pm 2*	46 \pm 2*	112 \pm 2	86 \pm 8
control		32 \pm 1	46 \pm 5	34 \pm 6	116 \pm 5	80 \pm 4
72T ₄ +0GH		28 \pm 2*	52 \pm 4	35 \pm 2	117 \pm 3	77 \pm 5
control		35 \pm 7	45 \pm 5	37 \pm 2	115 \pm 4	72 \pm 4
72T ₄ +5GH		32 \pm 6	50 \pm 1	34 \pm 2	120 \pm 4	80 \pm 4*
control		30 \pm 2	53 \pm 4	36 \pm 5	122 \pm 3	83 \pm 5
72T ₄ +10GH		31 \pm 2	46 \pm 6	36 \pm 2	112 \pm 6*	67 \pm 3*
control		32 \pm 6	48 \pm 4	35 \pm 4	114 \pm 4	82 \pm 2
72T ₄ +15GH		27 \pm 3	50 \pm 1	35 \pm 2	109 \pm 2	73 \pm 11
control		35 \pm 1	48 \pm 6	35 \pm 5	119 \pm 5	86 \pm 2
72T ₄ +30GH		33 \pm 2	46 \pm 1	38 \pm 4	107 \pm 3*	73 \pm 3*
control		31 \pm 1	50 \pm 2	37 \pm 6	121 \pm 3	82 \pm 8
72T ₄ +60GH		29 \pm 2	49 \pm 1	36 \pm 4	111 \pm 6*	78 \pm 10

Results are expressed as mean \pm s.d. (N=3); * = P < 0.05 as compared to relevant control values (C=control).

Figure 43. Thus 10^{-8} M thyroxine has no significant effects on enzyme activity, which are not markedly affected by further incubation with growth hormone.

3.5 DEXAMETHASONE AND THYROXINE

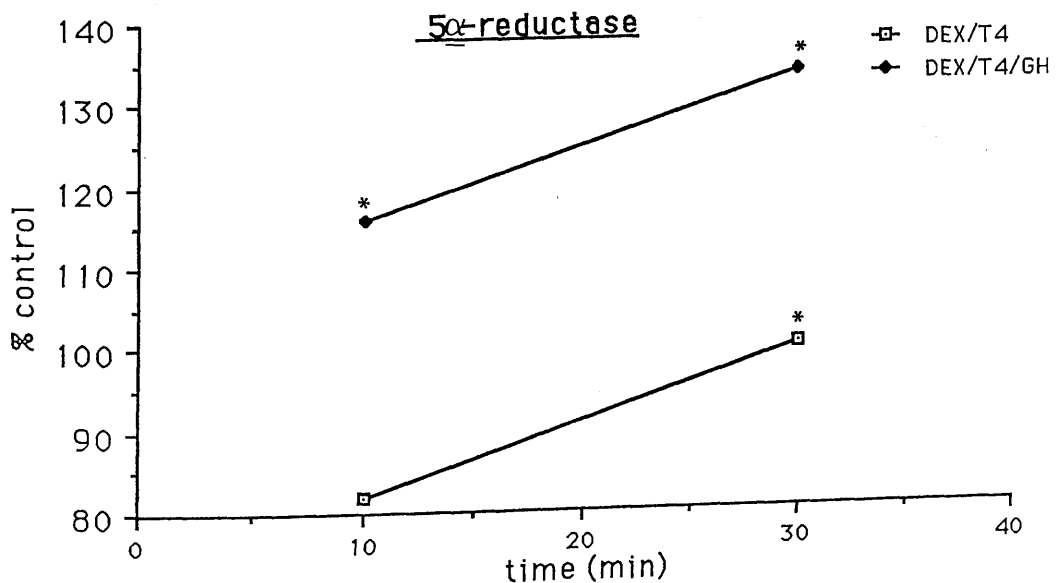
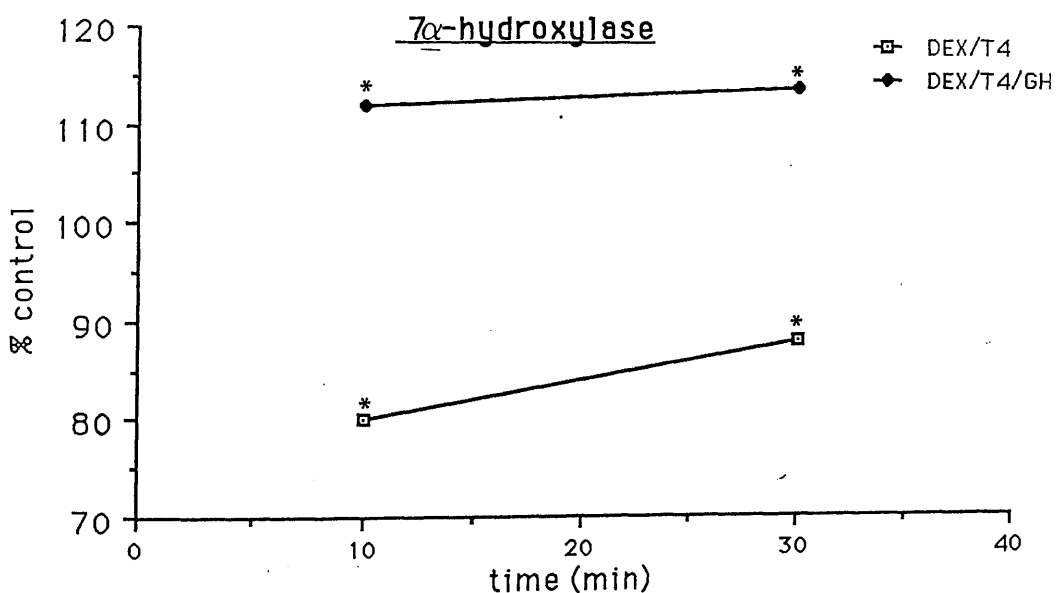
3.5.1 EFFECTS OF INCUBATION WITH 10^{-9} M GROWTH HORMONE IN THE PRESENCE OF 10^{-8} M DEXAMETHASONE AND 10^{-6} M THYROXINE

Hepatocytes were preincubated with 10^{-9} M growth hormone in the presence of both 10^{-8} M dexamethasone and 10^{-6} M thyroxine over two incubation times (Table 24).

As is illustrated in Figure 44A and 44B, the female-specific enzyme activities 7 α -hydroxylase and 5 α -reductase were markedly increased at both 10 and 30 minutes of incubation, whereas enzyme activity was significantly decreased in the absence of growth hormone. The male-specific enzymes 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase (Figures 44C, 44D and 44E respectively) were significantly decreased with respect to the response observed in the absence of growth hormone (which remained at basal levels).

Thus the feminising effects of growth hormone shown in vivo by numerous workers (see Introduction) and typified by an increase in female-specific and a decrease in male-specific activities has been demonstrated above in the presence of both dexamethasone and thyroxine.

FIGURE 44 : Effects of preincubation with 10^{-6} M THYROXINE and 10^{-8} M DEXAMETHASONE in the absence and presence of 10^{-9} M GROWTH HORMONE for 10 and 30 minutes on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 24



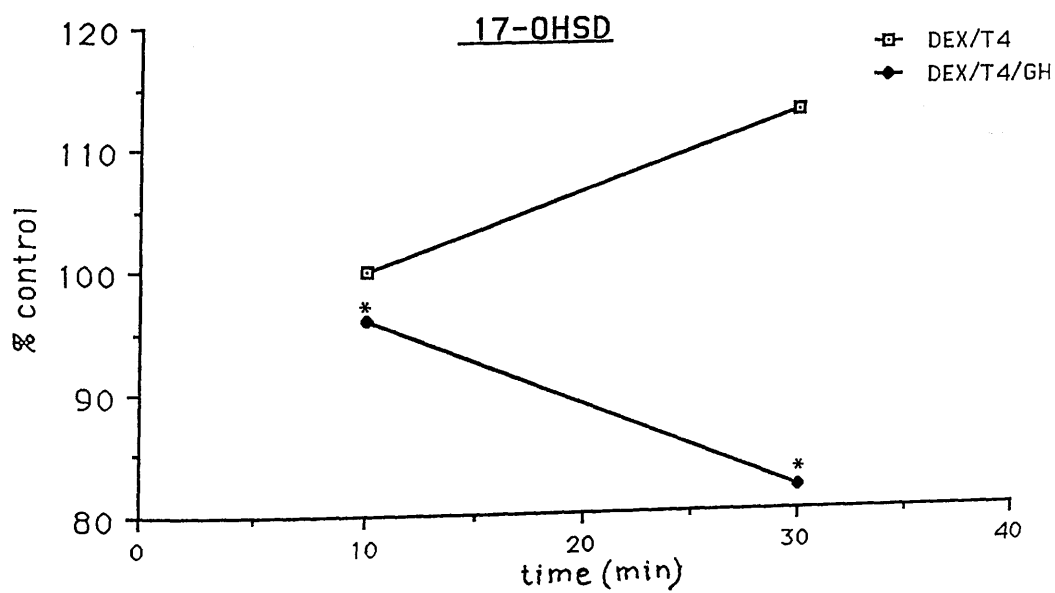
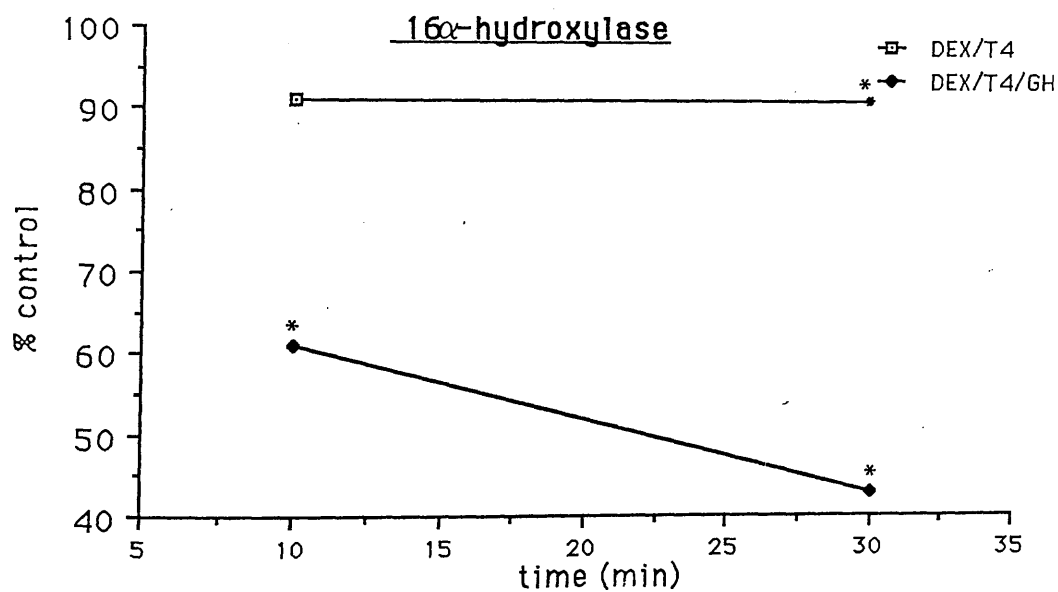
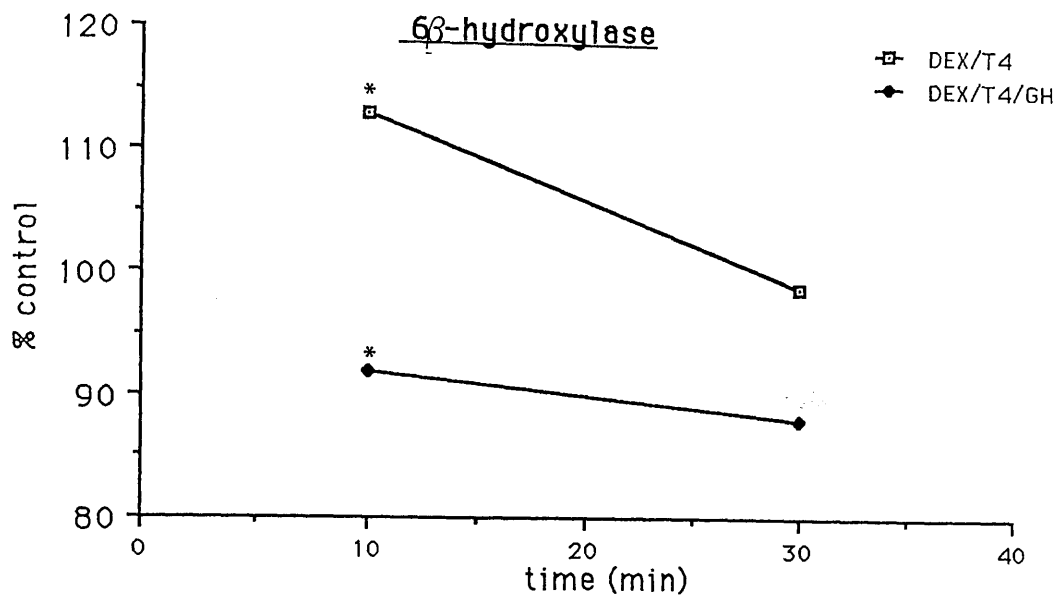


TABLE 24 : Effects of preincubation with 10^{-6} M THYROXINE and 10^{-8} M DEXAMETHASONE in the absence and presence of 10^{-9} M GROWTH HORMONE for 10 and 30 minutes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

TREATMENT	Enzyme activities (pmoles/minute/million cells)				
	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
10 C	51 \pm 6	38 \pm 2	38 \pm 2	87 \pm 3	135 \pm 5
10 T ₄ /DEX	41 \pm 5*	44 \pm 2*	34 \pm 4	87 \pm 2	111 \pm 1*
30 C	40 \pm 3	49 \pm 4	57 \pm 2	101 \pm 4	106 \pm 11
30 T ₄ /DEX	33 \pm 2*	54 \pm 4	41 \pm 3*	94 \pm 5	134 \pm 4*
10 C	38 \pm 2	54 \pm 1	45 \pm 4	83 \pm 6	134 \pm 10
10 T ₄ /D+GH	45 \pm 1*	45 \pm 3*	35 \pm 1*	97 \pm 3*	123 \pm 3
30 C	35 \pm 2	61 \pm 6	82 \pm 4	110 \pm 5	110 \pm 12
30 T ₄ /D+GH	40 \pm 3*	54 \pm 3	35 \pm 2*	90 \pm 2*	148 \pm 5*

Results are expressed as mean \pm s.d. (N=3); * = P < 0.05 as compared to relevant control values (C=control).

3.5.2 A COMPARISON OF THE EFFECTS OF 10^{-8} M AND 10^{-9} M
GROWTH HORMONE IN THE PRESENCE OF 10^{-8} M DEXAMETHASONE AND
 10^{-6} M THYROXINE

The experiment was essentially identical to that in section 3.5.1 except that the effects of a higher dose of growth hormone were also examined (Table 25).

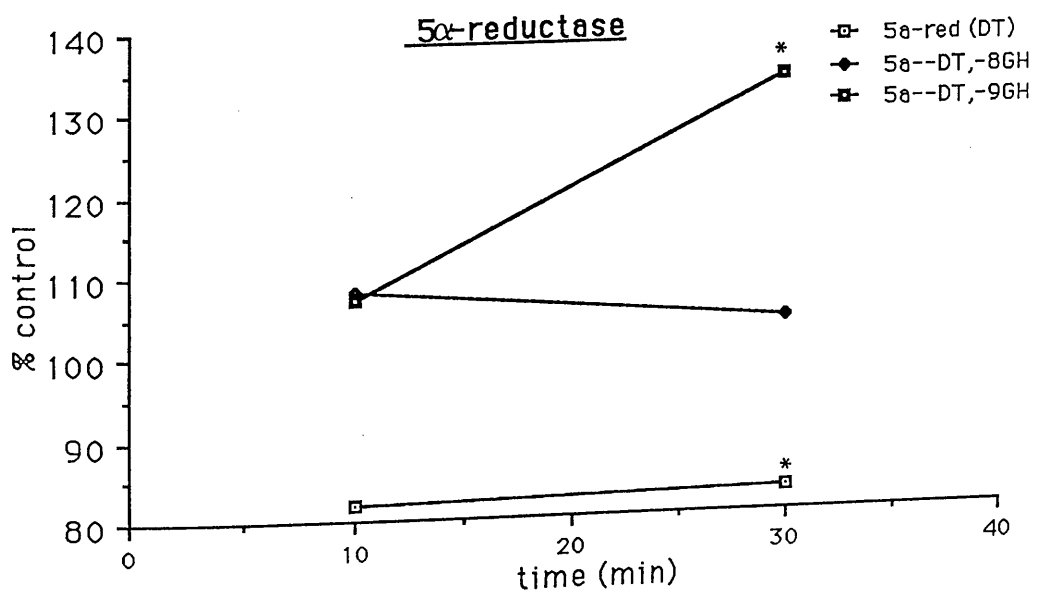
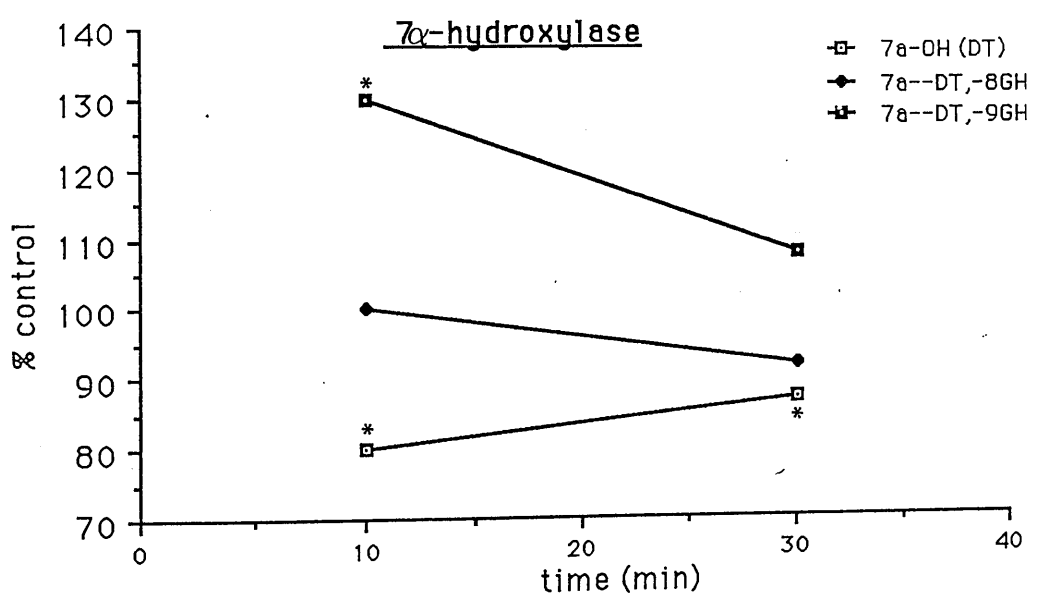
7 α -hydroxylase activity (Figure 45A) was significantly increased upon incubation with growth hormone in the presence of dexamethasone and thyroxine, the effect being more pronounced with 10^{-9} M than with 10^{-8} M growth hormone. This increase in enzyme activity was, however, not significant at 30 minutes of incubation.

5 α -reductase activity (Figure 45B) was significantly reduced upon incubation with dexamethasone and thyroxine, and increased when growth hormone was included in the incubation medium. Again this increase was more marked upon incubation with 10^{-9} M than with 10^{-8} M growth hormone, the effect being more obvious after 30 minutes of incubation.

The male-specific enzyme activities, 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase (Figures 45C, 45D and 45E respectively), were all significantly decreased upon incubation with growth hormone in the presence of dexamethasone and thyroxine. The effect of growth hormone was, however, greater at 10^{-8} M than at 10^{-9} M, in contrast to the situation seen with the female-specific enzymes above.

Thus growth hormone exhibited a feminisation of steroid metabolism when incubated together with dexamethasone and

FIGURE 45 : A comparison of the effects preincubation with 10^{-6} M THYROXINE and 10^{-8} M DEXAMETHASONE in the absence and presence of 10^{-9} M or 10^{-8} M GROWTH HORMONE for 10 and 30 minutes on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 25



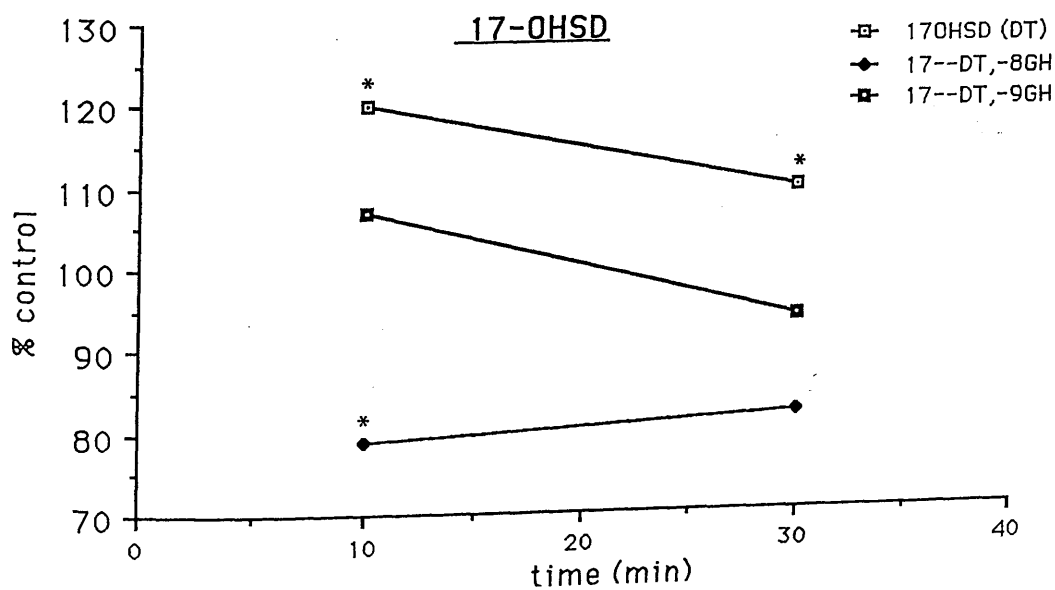
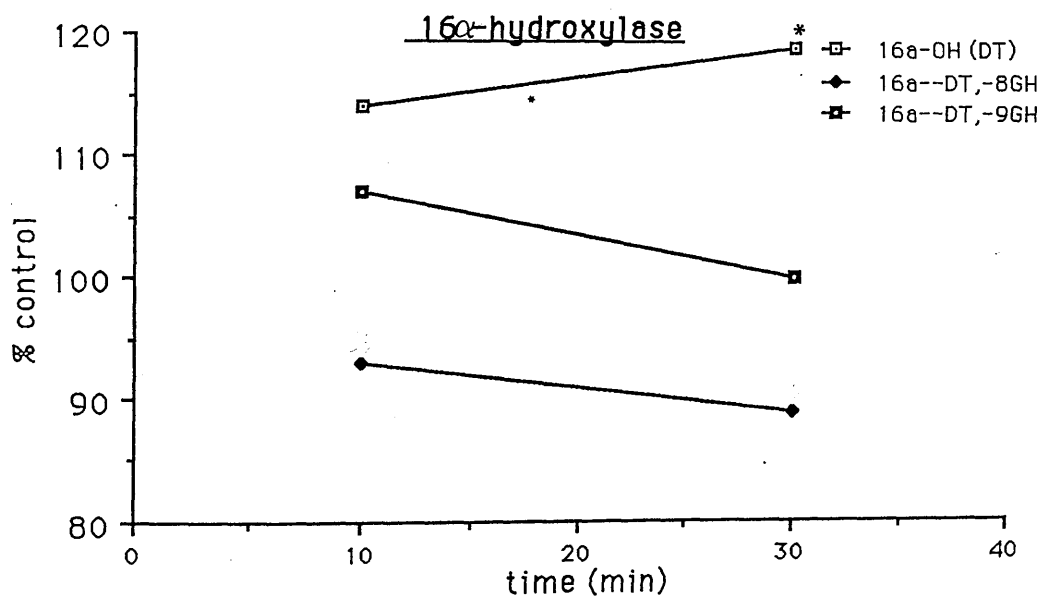
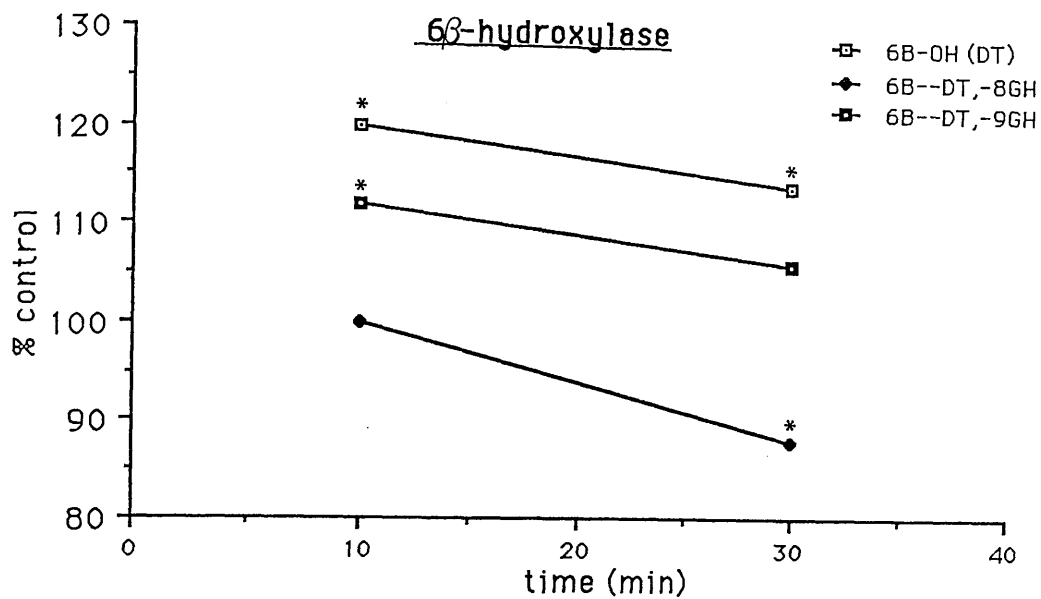


TABLE 25 : Effects of preincubation with 10^{-6} M THYROXINE and 10^{-8} M DEXAMETHASONE in the absence and presence of 10^{-9} M or 10^{-8} M GROWTH HORMONE for 10 and 30 minutes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

TREATMENT	Enzyme activities (pmoles/minute/million cells)				
	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
10 C	50 \pm 3	70 \pm 4	70 \pm 10	80 \pm 6	76 \pm 12
10 DT ₄	40 \pm 3*	60 \pm 3*	80 \pm 7	90 \pm 4*	62 \pm 6
10 C	50 \pm 6	80 \pm 5	70 \pm 7	70 \pm 5	85 \pm 9
10 DT ₄ /-8GH	50 \pm 4	80 \pm 7	65 \pm 4	55 \pm 3*	92 \pm 5
10 DT ₄ /-9GH	65 \pm 2*	90 \pm 8*	75 \pm 4	75 \pm 4	91 \pm 2
30 C	75 \pm 5	70 \pm 3	80 \pm 8	80 \pm 10	169 \pm 4
30 DT ₄	65 \pm 3*	80 \pm 4*	95 \pm 10*	110 \pm 5*	75 \pm 4*
30 C	60 \pm 4	85 \pm 7	90 \pm 10	80 \pm 4	92 \pm 8
30 DT ₄ /-8GH	65 \pm 5	75 \pm 5*	80 \pm 7	75 \pm 7	96 \pm 8
30 DT ₄ /-9GH	55 \pm 6	90 \pm 3	90 \pm 3	65 \pm 4*	123 \pm 9*

Results are expressed as mean \pm s.d. (N=3); * = $P < 0.05$ as compared to relevant control values (C=control).

thyroxine. This effect of growth hormone was more effective at 10^{-9}M on female-specific enzymes, but 10^{-8}M growth hormone appeared to be more potent with male-specific enzymes.

3.5.3 EFFECTS OF INCUBATION WITH 10^{-9}M GROWTH HORMONE ON HEPATOCYTES PRETREATED WITH 10^{-8}M DEXAMETHASONE AND 10^{-6}M THYROXINE FOR 24, 48 AND 72 HOURS.

Hepatocytes were cultured with 10^{-8}M dexamethasone and 10^{-6}M thyroxine for 24, 48 and 72 hours, as described in section 2.5.2.

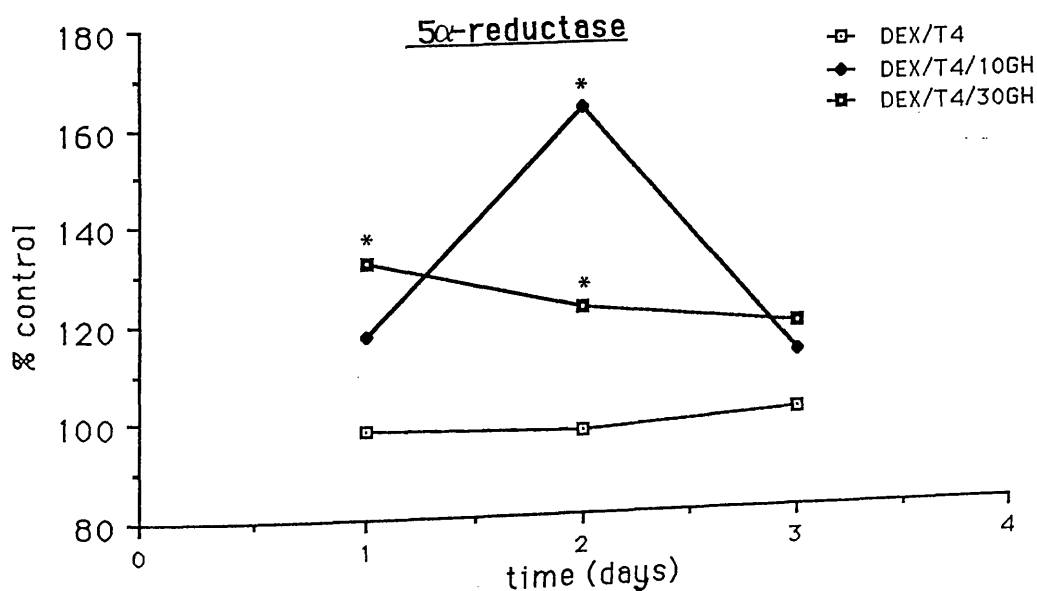
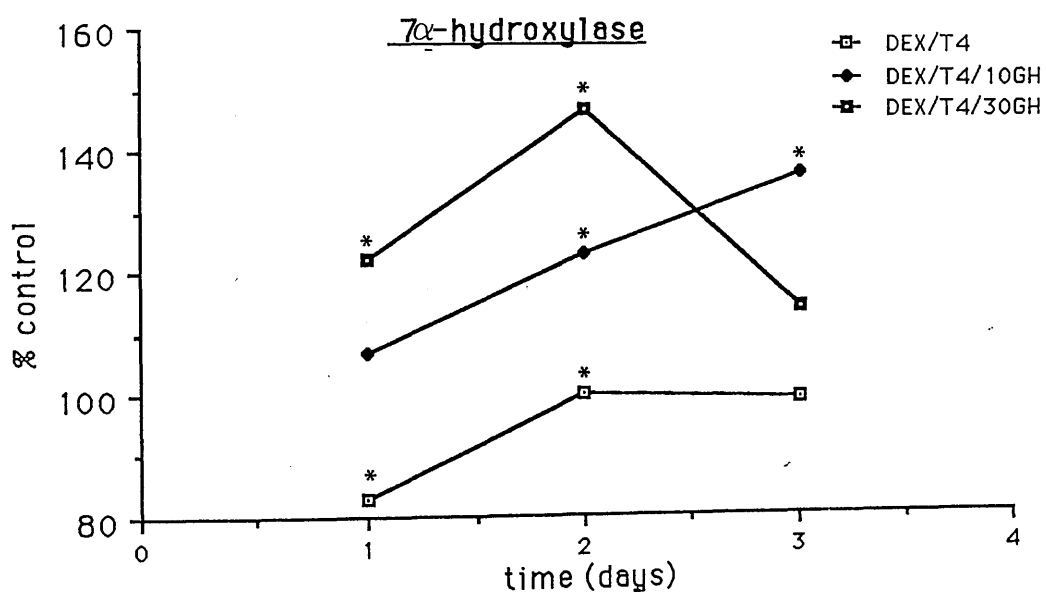
Table 26 shows that incubation of hepatocytes with a combination of dexamethasone and thyroxine over 24, 48 and 72 hours had no significant effects on enzyme activity. Further incubation of pretreated hepatocytes with 10^{-9}M growth hormone for 10 and 30 minutes showed that 7 α -hydroxylase was markedly increased with respect to hepatocytes not exposed to growth hormone (Figure 46A).

A significant increase was observed in 5 α -reductase activity (Figure 46B) upon incubation of pretreated hepatocytes with growth hormone, although this effect was lost with hepatocytes pretreated for 72 hours.

Incubation of pretreated hepatocytes with 10^{-9}M growth hormone caused a significant decrease in the male-specific 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase activities (Figures 46C, 46D and 46E respectively), although this effect was not significant with 16 α -hydroxylase activity at 48h or with 17-oxosteroid

FIGURE 46 : Effects of preincubation with 10^{-6} M THYROXINE and 10^{-8} M DEXAMETHASONE over 24, 48 and 72 hours followed by incubation with 10^{-9} M GROWTH HORMONE for 10 and 30 minutes on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 26



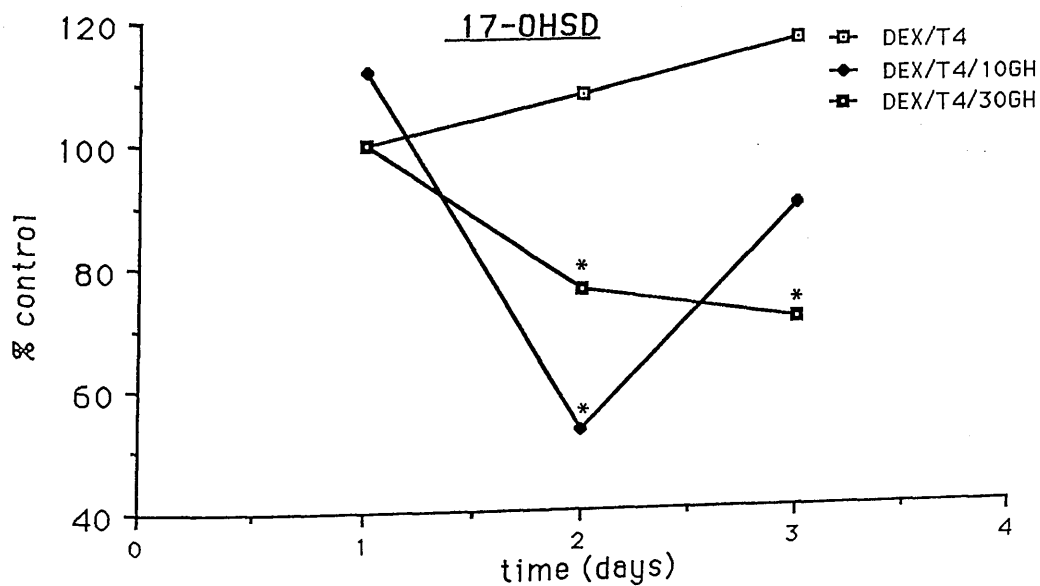
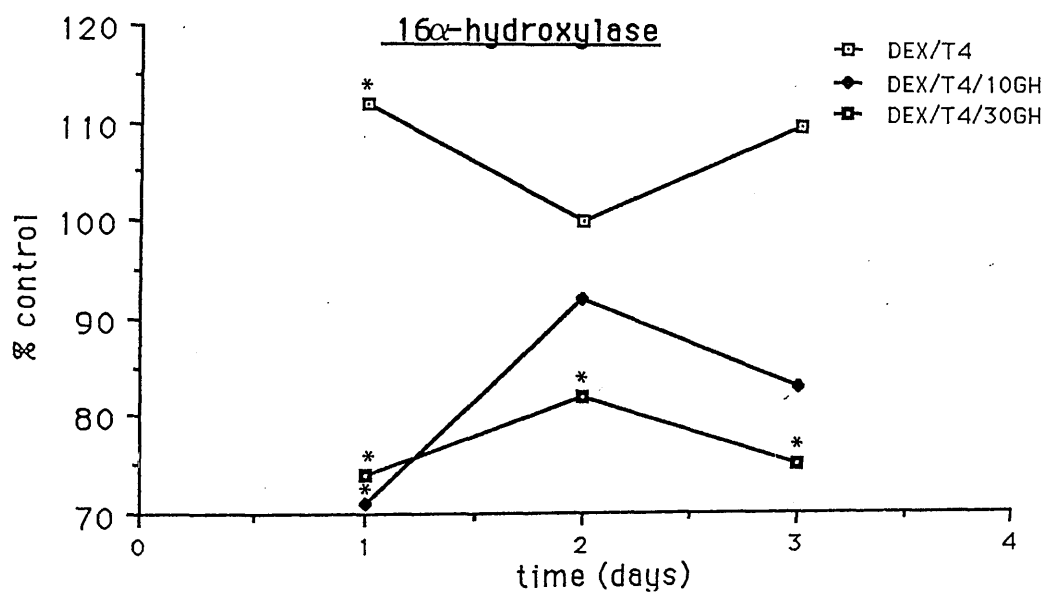
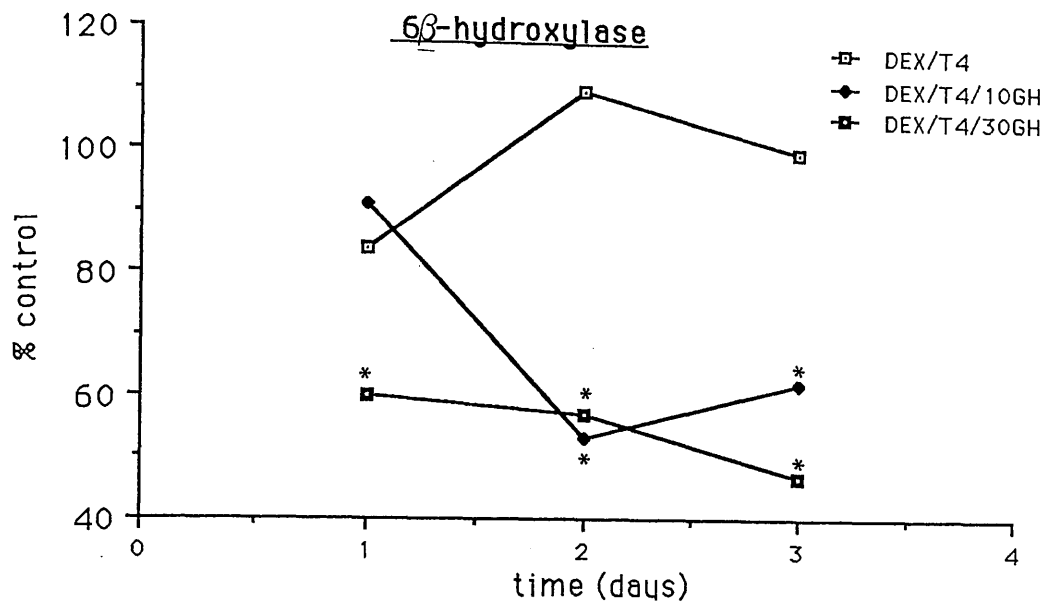


TABLE 26 : Effects of preincubation with 10^{-6} M THYROXINE and 10^{-8} M DEXAMETHASONE over 24, 48 and 72h followed by incubation with 10^{-9} M GROWTH HORMONE for 10 and 30 minutes on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

Enzyme activities (pmoles/minute/million cells)					
TREATMENT	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
24C	24 \pm 2	25 \pm 1	25 \pm 2	22 \pm 2	125 \pm 18
24DexT ₄	20 \pm 1*	26 \pm 1	28 \pm 1*	21 \pm 1	122 \pm 10
control	30 \pm 2	32 \pm 4	28 \pm 4	32 \pm 2	60 \pm 7
24DT ₄ +10GH	32 \pm 3	29 \pm 2	20 \pm 4*	36 \pm 4	70 \pm 9
control	36 \pm 3	30 \pm 2	38 \pm 1	44 \pm 3	60 \pm 5
24DT ₄ +30GH	44 \pm 5*	18 \pm 2*	28 \pm 3*	44 \pm 4	79 \pm 4*
48C	20 \pm 2	20 \pm 1	27 \pm 1	13 \pm 1	137 \pm 14
48DexT ₄	16 \pm 1*	22 \pm 2	27 \pm 2	14 \pm 1	133 \pm 15
control	26 \pm 1	38 \pm 1	36 \pm 2	38 \pm 3	57 \pm 4
48DT ₄ +10GH	32 \pm 3*	20 \pm 1*	33 \pm 4	20 \pm 3*	93 \pm 6*
control	28 \pm 2	28 \pm 2	34 \pm 3	34 \pm 4	65 \pm 5
48DT ₄ +30GH	38 \pm 6*	16 \pm 2*	28 \pm 3*	26 \pm 2*	79 \pm 2*
72C	15 \pm 1	25 \pm 2	21 \pm 2	18 \pm 2	164 \pm 12
72DexT ₄	16 \pm 1	25 \pm 3	23 \pm 2	21 \pm 2	164 \pm 15
control	14 \pm 1	24 \pm 3	24 \pm 3	20 \pm 2	41 \pm 4
72DT ₄ +10GH	19 \pm 2*	15 \pm 1*	20 \pm 2	18 \pm 1	46 \pm 3
control	14 \pm 2	19 \pm 2	28 \pm 4	21 \pm 1	32 \pm 5
72DT ₄ +30GH	16 \pm 2	9 \pm 1*	21 \pm 2*	15 \pm 2*	38 \pm 2

Results are expressed as mean \pm s.d. (N=3); *= $P < 0.05$ as compared to relevant control values (C=control).

oxidoreductase activity at 24h.

Thus the feminising effects of growth hormone are, as with the short incubation experiments, manifested by hepatocytes pretreated with dexamethasone and thyroxine over 24, 48 and 72 hours.

3.6 INSULIN MEDIATOR

3.6.1 EFFECT OF INCUBATION OF RAT LIVER MITOPLASTS WITH 10^{-11} M- 10^{-6} M INSULIN ON MITOCHONDRIAL PYRUVATE DEHYDROGENASE

Rat liver mitoplasts, highly enriched in pyruvate dehydrogenase activity, were prepared as detailed in section 2.3, and incubated with insulin prior to assaying for stimulation of enzyme activity (section 2.10).

As is shown in Table 27, and illustrated in Figure 47, insulin significantly increased pyruvate dehydrogenase activity at all concentrations tested, except with 10^{-6} M insulin. This increase in enzyme activity was dose-dependent, and exhibited maximum stimulation upon incubation with 10^{-9} M insulin.

FIGURE 47 : Dose-dependent effects of incubation of rat liver mitoplasts with 10^{-11} M- 10^{-6} M INSULIN on mitochondrial pyruvate dehydrogenase activity. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 27.

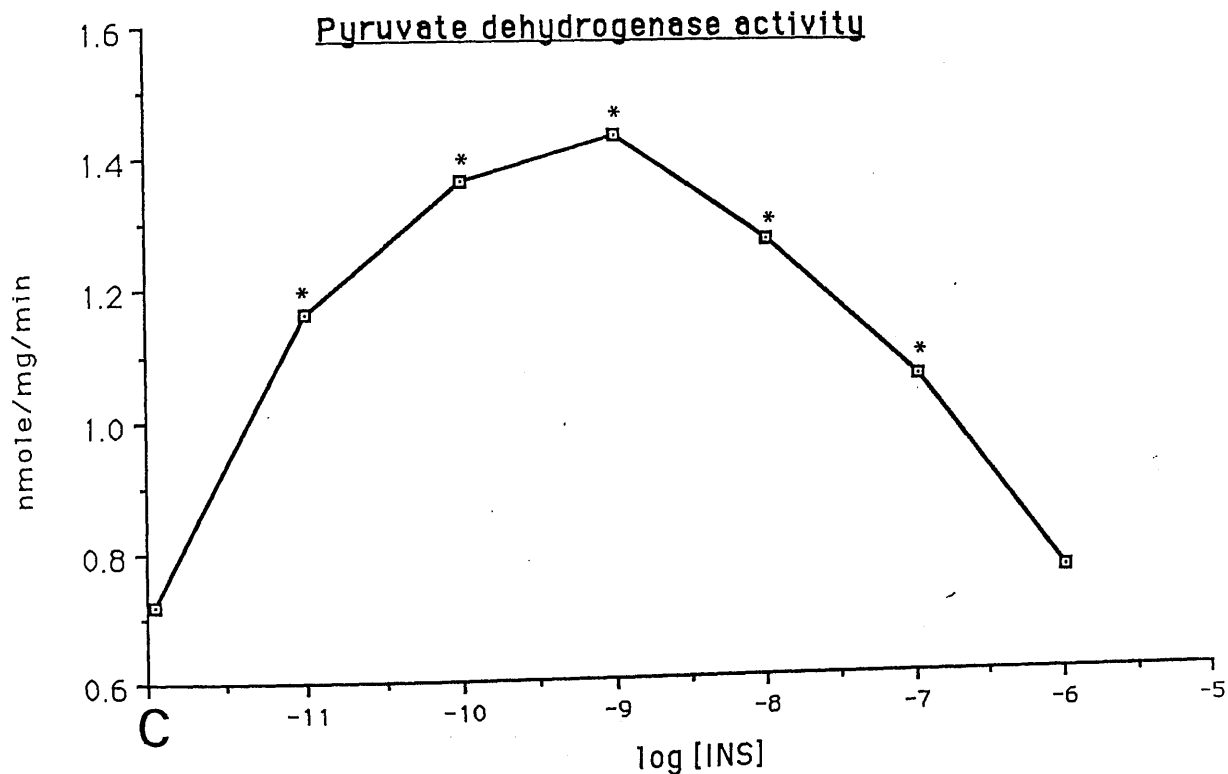


TABLE 27 : Dose-dependent effects of incubation of rat liver mitoplasts with 10^{-11} - 10^{-6} M INSULIN on mitochondrial pyruvate dehydrogenase activity.

<u>INSULIN CONC^N.</u>	<u>Enzyme activity (nmoles/mg protein/minute)</u>
Control (A)	0.72 \pm 0.089
10^{-11} M (B)	1.16 \pm 0.160*
10^{-10} M (C)	1.36 \pm 0.059*
10^{-9} M (D)	1.43 \pm 0.132*
10^{-8} M (E)	1.27 \pm 0.033*
10^{-7} M (F)	1.06 \pm 0.039*
10^{-6} M (G)	0.76 \pm 0.045

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to control.

Duncan's Multiple Range Test :

A G F B E C D

3.6.2 EFFECT OF INCUBATION OF RAT LIVER MITOPLASTS WITH
VARYING DOSES OF INSULIN MEDIATOR PRODUCED USING 10^{-9} M
INSULIN

Rat liver mitoplasts were incubated with various dilutions of the insulin mediator extract prepared from hepatocytes incubated with 10^{-9} M insulin (Section 2.8.2).

As is shown in Table 28 and in Figure 48, incubation with the insulin mediator extract significantly increased pyruvate dehydrogenase activity at all dilutions tested. This stimulation of enzyme activity was maximal using a 1:100 dilution of the original insulin mediator extract. Further ten-fold dilutions still resulted in a significant stimulation of enzyme activity, although not as marked as that caused by incubation with a 1:100 dilution. Incubation with more concentrated extracts also resulted in a stimulation of enzyme activity but exhibited a "tailing-off" effect as mediator amount was gradually increased.

Thus incubation with various dilutions of a 10^{-9} M insulin mediator extract resulted in a dose-dependent increase in pyruvate dehydrogenase activity and a certain degree of amplification of the insulin response was exhibited in that a 1:100 dilution caused maximum stimulation. It therefore appears that the insulin mediator extract prepared is active in stimulating pyruvate dehydrogenase activity and is therefore similar to the insulin mediator described by other workers (Suzuki et al 1987).

FIGURE 48 : Dose-dependent effects of incubation of rat liver mitoplasts with various dilutions of the INSULIN MEDIATOR extract prepared using 10^{-9} M INSULIN on mitochondrial pyruvate dehydrogenase activity. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 28

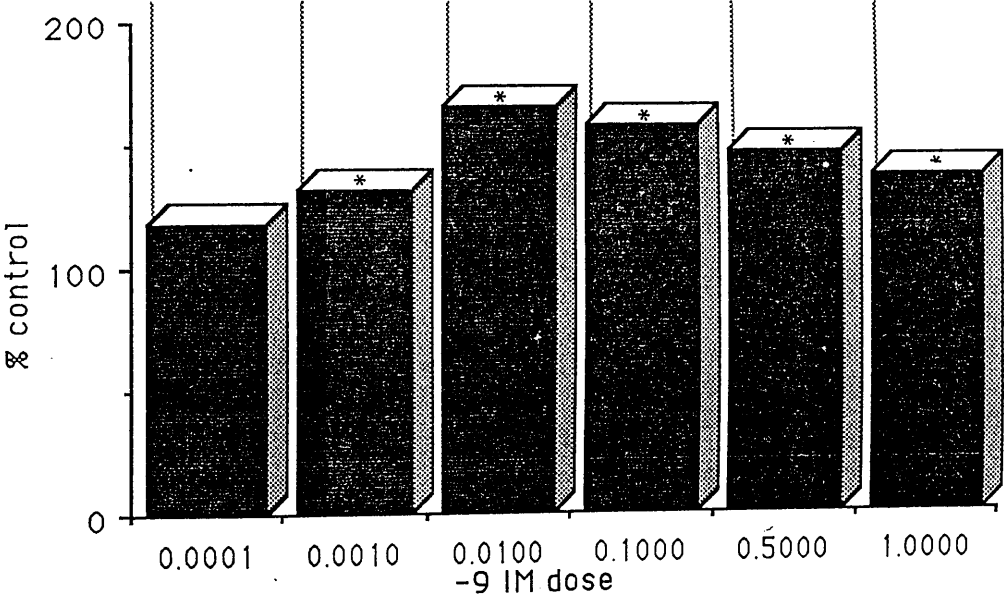


TABLE 28 : Dose-dependent effects of incubation of rat liver mitoplasts with various dilutions of the INSULIN MEDIATOR extract prepared using 10^{-9} M INSULIN on mitochondrial pyruvate dehydrogenase activity.

<u>IM dilution</u>	<u>Enzyme activity (nmoles/mg protein/minute)</u>
control	0.68 ± 0.087
$1:10^{-4}$	0.80 ± 0.080
control	0.76 ± 0.099
$1:10^{-3}$	$1.00 \pm 0.096^*$
control	1.34 ± 0.141
$1:10^{-2}$	$2.22 \pm 0.118^*$
control	1.98 ± 0.039
$1:10$	$3.12 \pm 0.077^*$
control	2.46 ± 0.030
$1:2$	$3.64 \pm 0.101^*$
control	2.60 ± 0.119
$1:1$	$3.58 \pm 0.178^*$

Results are expressed as MEAN \pm S.D. (N=3); $^*=P<0.05$ as compared to relevant control values.

3.6.3 INSULIN MEDIATOR DOSE-RESPONSE CURVE

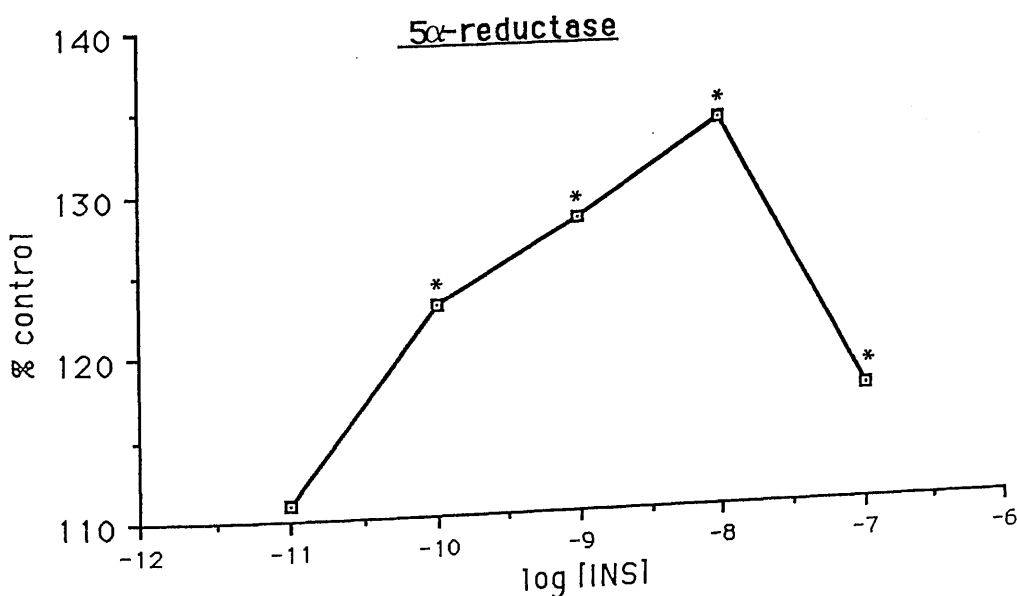
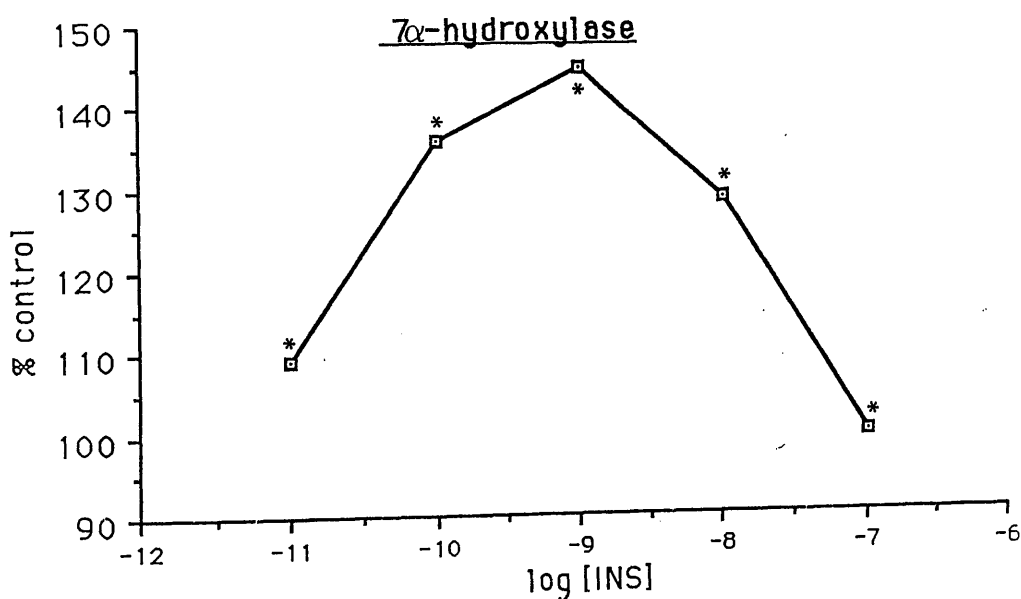
An insulin mediator extract was prepared by treating hepatocytes with 10^{-11}M - 10^{-7}M insulin, as described in section 2.8.2. Hepatocytes were incubated with this extract for 30 minutes (section 2.9.1) prior to assaying for steroid metabolism.

Table 29 shows that the effects of the insulin mediator produced in response to different doses of insulin exhibits dose-related effects on enzyme activity. As is illustrated in Figure 49A, 49D and 49E respectively, the stimulation of 7 α -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase activities is maximal upon incubation with the insulin mediator extract prepared in response to 10^{-9}M insulin, and with 10^{-8}M insulin mediator for 6 β -hydroxylase and 5 α -reductase activities (Figure 49C and 49B respectively). Enzyme activity is significantly reduced upon incubation with insulin mediator prepared using supraphysiological concentrations of insulin.

Thus the above data illustrates that the effects of the insulin mediator extract prepared using a range of insulin concentrations exhibit dose-dependency, and that this dose-related response is reminiscent of that observed upon incubation of hepatocytes with various concentrations of insulin.

Since physiological concentrations of insulin displayed maximum effects, insulin mediator prepared using 10^{-9}M insulin is used for future assays, unless otherwise indicated.

FIGURE 49 : Dose-dependent effects of 30 minute incubation of hepatocytes with 1:1 INSULIN MEDIATOR extract prepared using 10^{-11}M - 10^{-7}M INSULIN on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17 -oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 29



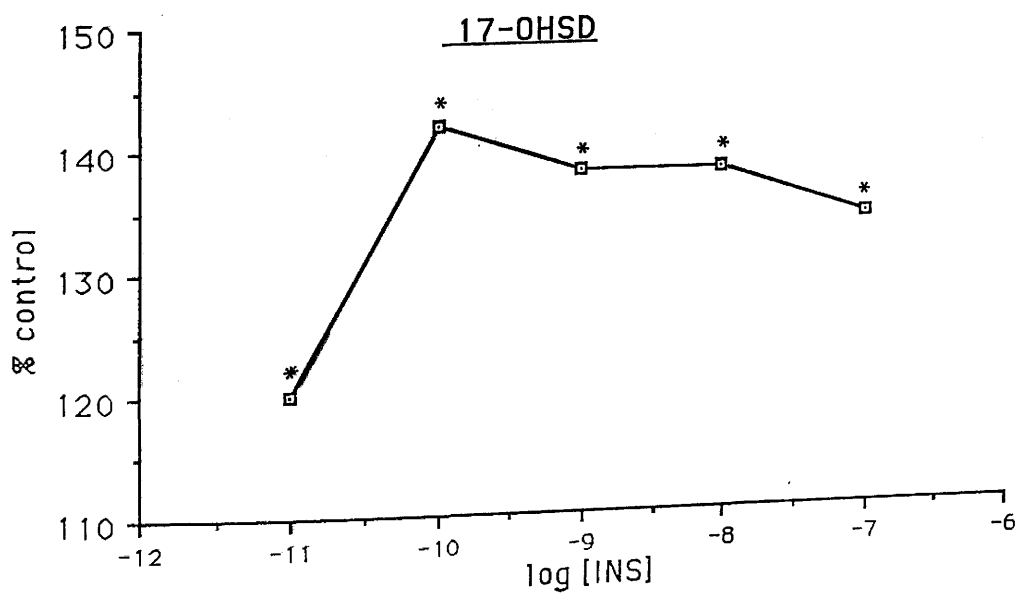
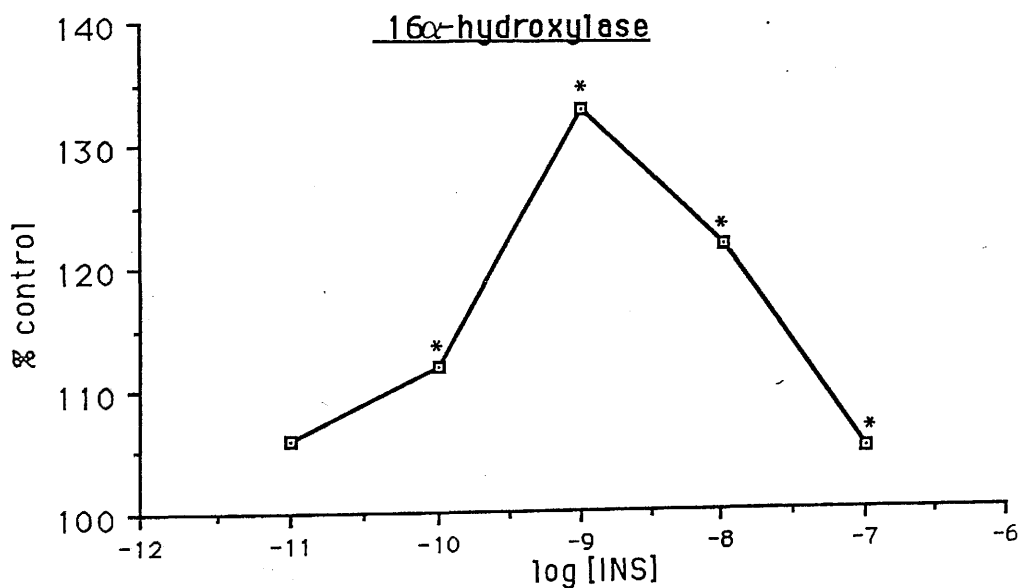
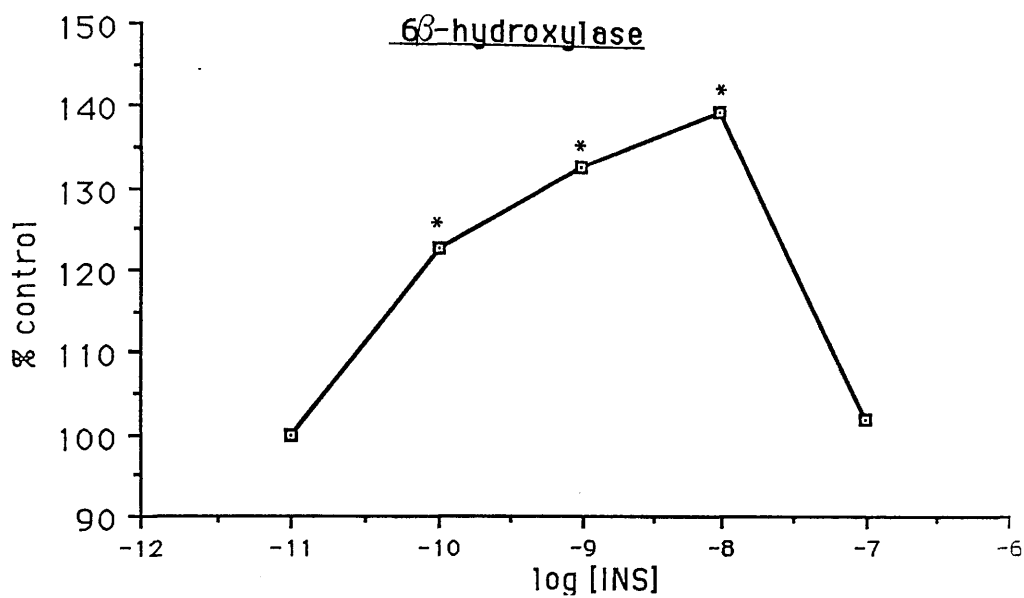


TABLE 29 : Dose-dependent effects of 30 minute incubation of male rat hepatocytes with 1:1 INSULIN MEDIATOR extract prepared using 10^{-11}M - 10^{-7}M INSULIN on 7α -, 6β -, 16α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5α -reductase activities.

Enzyme activities (pmoles/minute/million cells)					
TREATMENT	7α -OHase	6β -OHase	16α -OHase	17-OHSD	5α -red
Control (A)	47±1	59±3	62±2	119±4	174±7
10^{-11}M (B)	62±6*	59±1	66±7	143±11*	175±2
10^{-10}M (C)	70±5*	73±10*	69±2*	169±24*	207±14*
10^{-9}M (D)	68±5*	79±6*	82±3*	164±26*	223±29*
10^{-8}M (E)	72±1*	83±4*	75±8*	164±15*	231±11*
10^{-7}M (F)	66±10*	61±1	65±1*	159±18*	211±17*

Results are expressed as MEAN ± S.D. (N=3); *=P<0.05 as compared to control.

Duncan's Multiple Range Test :

7α -OHase	A	B	F	D	C	E
6β -OHase	A	B	F	C	D	E
16α -OHase	A	F	B	C	E	D
17-OHSD	A	B	F	D	E	C
5α -red	A	B	F	C	D	E

3.6.4 TIME COURSE OF INSULIN MEDIATOR GENERATION

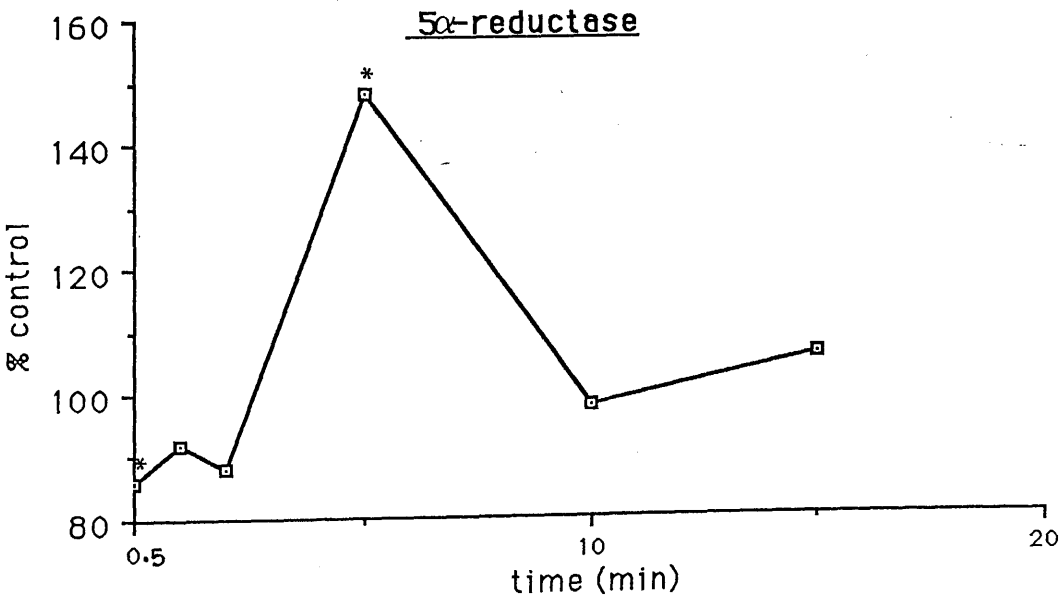
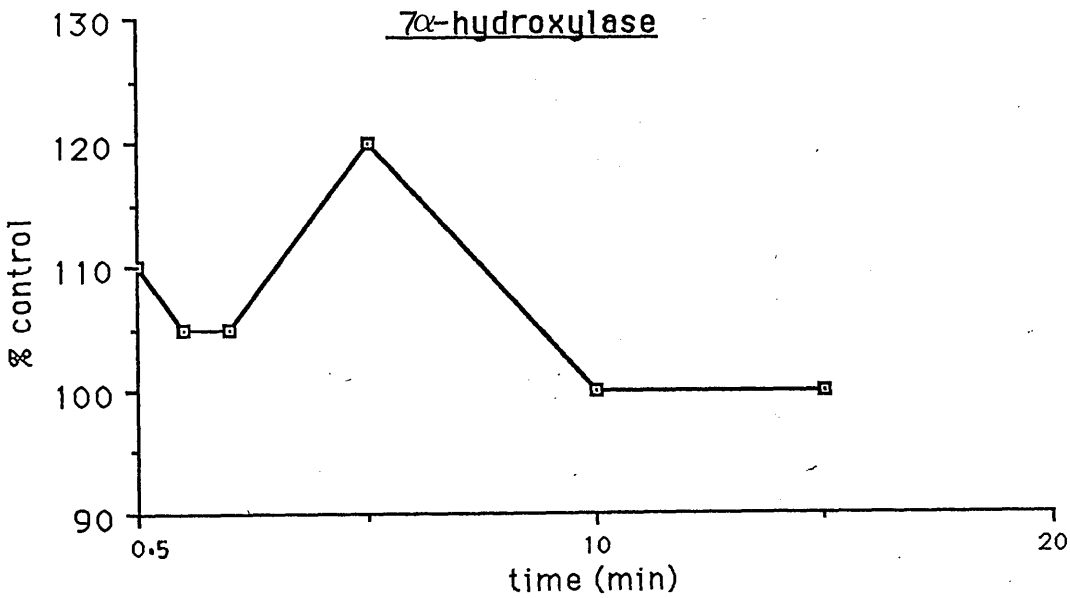
Hepatocytes were pretreated with 10^{-9} M insulin for 0.5, 1, 2, 5, 10 and 15 minutes in order to determine the time of incubation optimal for mediator production. Hepatocytes were then incubated with these insulin mediator extracts (1:2 and undiluted extract) for 30 minutes prior to assaying for steroid-metabolising activity (Table 30).

Figure 50 illustrates the effects of incubation of these extracts (1:2 dilution) on enzyme activities. Enzyme activity was low in each case upon incubation of hepatocytes with mediator produced by 0.5, 1 and 2 minutes exposure to insulin, and was rapidly increased to maximum levels with mediator produced by 5 minute exposure to insulin. Mediator produced by exposure to insulin for longer time periods appeared to be inactive, and enzyme activity remained at basal levels upon incubation with these extracts, as is shown in Figure 50.

Incubation with undiluted extract prepared as above for varying exposure times to insulin exhibited the same pattern of enzyme stimulation as was observed above (Table 30B). Again enzyme activity was stimulated non-specifically with mediator prepared after 5 minute exposure to insulin, as is shown in Figure 51, and relatively unchanged with other extracts. 5α -reductase and 17-oxosteroid oxidoreductase activities (Figures 51B and 51E respectively) did however remain elevated upon incubation with extracts prepared with 10 and 15 minute exposure to insulin.

Thus mediator prepared by incubation with insulin for 5

FIGURE 50 : Time course of generation of the INSULIN MEDIATOR extract (1:2 dilution) upon treatment of hepatocytes with 10^{-9} M INSULIN for 0.5 to 15 minutes on A) 7α -hydroxylase, B) 5α -reductase, C) 6β -hydroxylase, D) 16α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 30A.



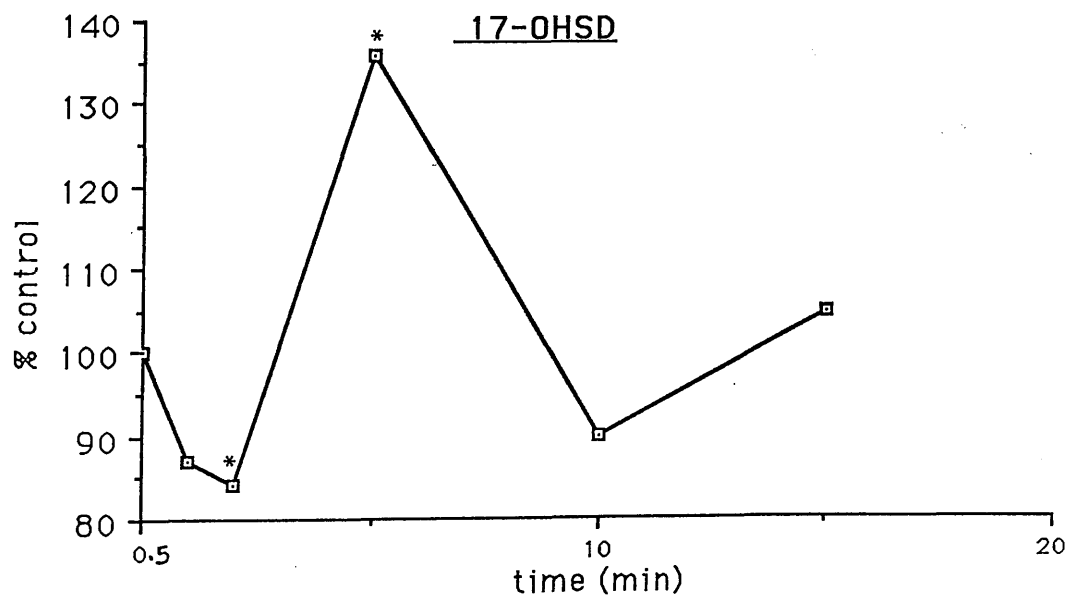
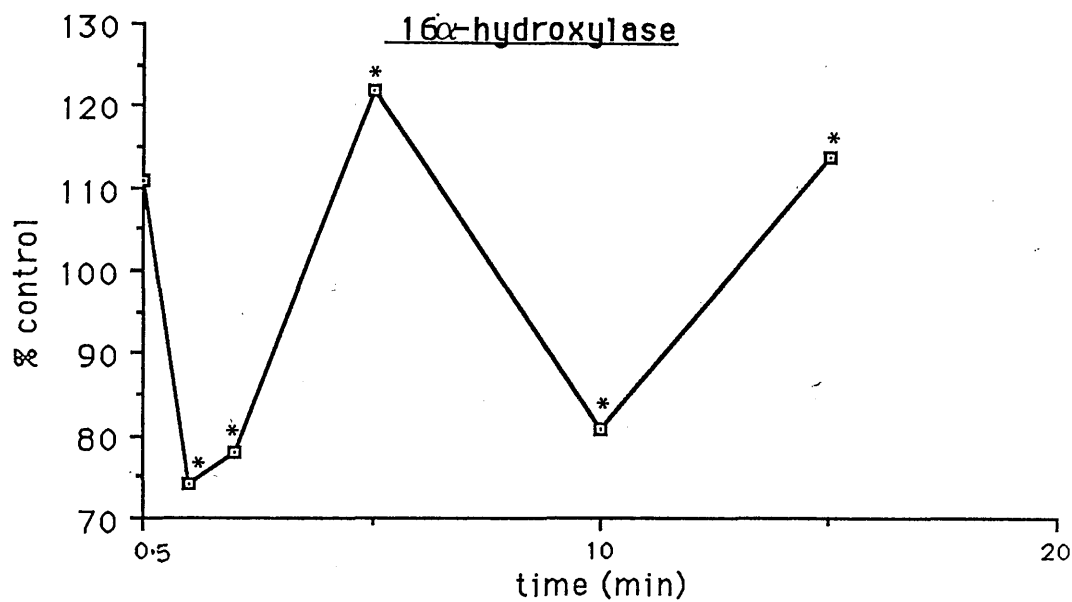
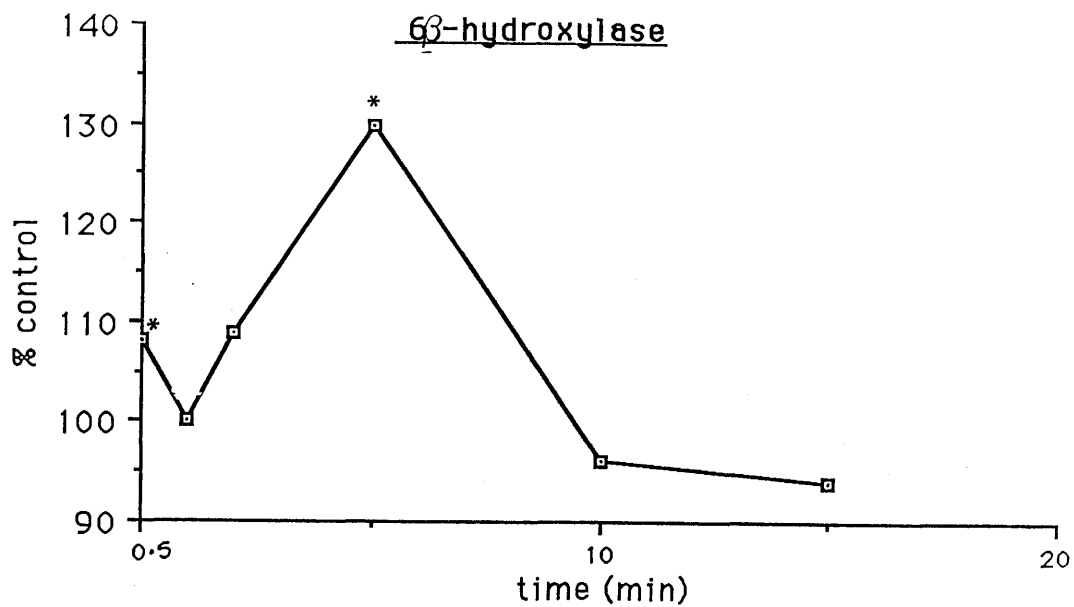
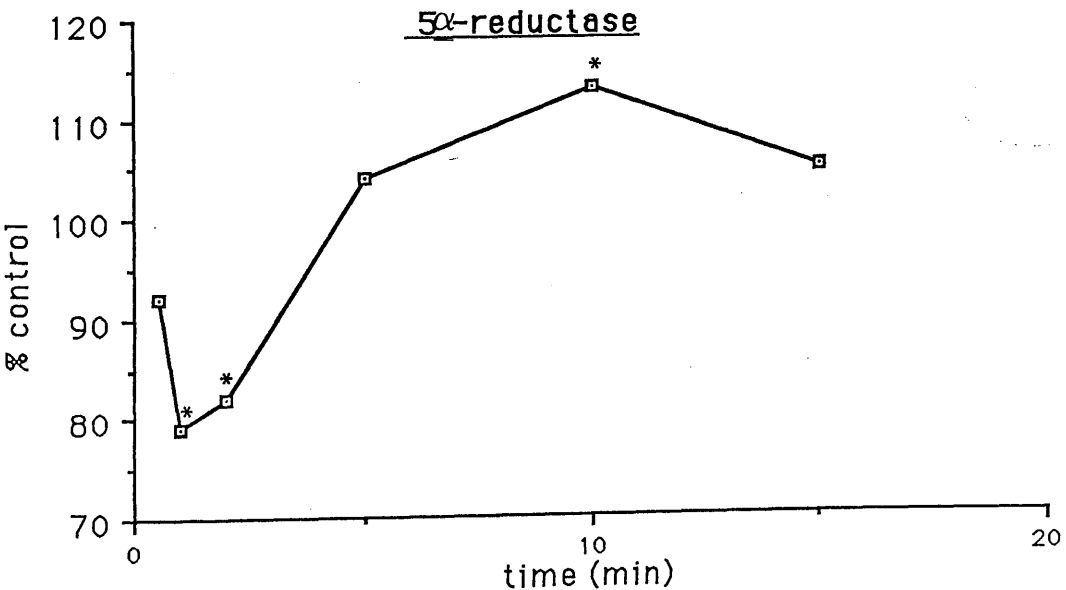
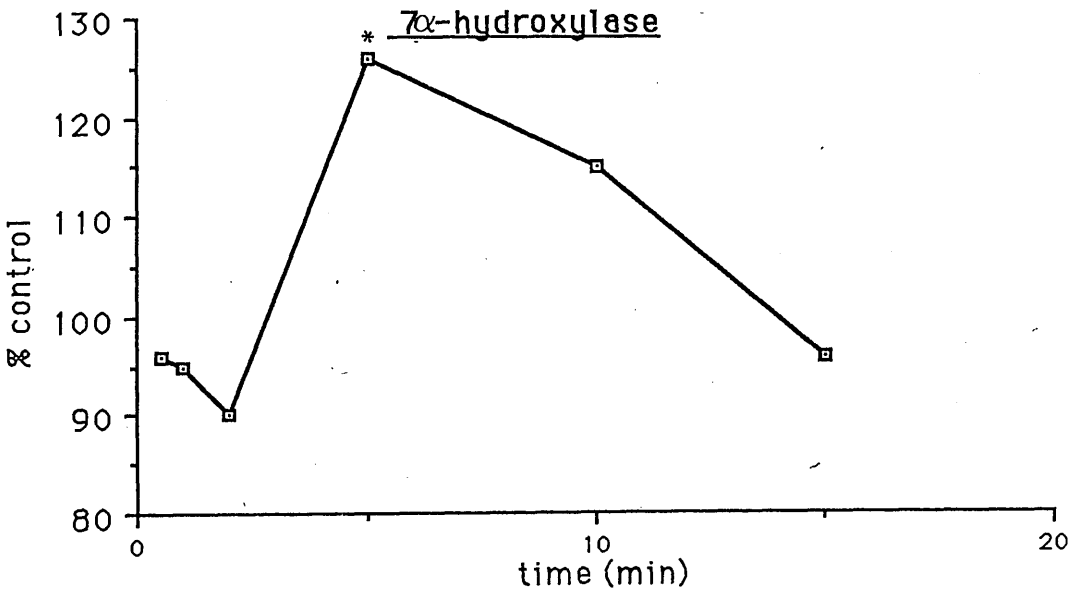


FIGURE 51 : Time course of generation of the INSULIN MEDIATOR extract (1:1) upon treatment of hepatocytes with 10^{-9} M INSULIN for 0.5 to 15 minutes on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 30B.



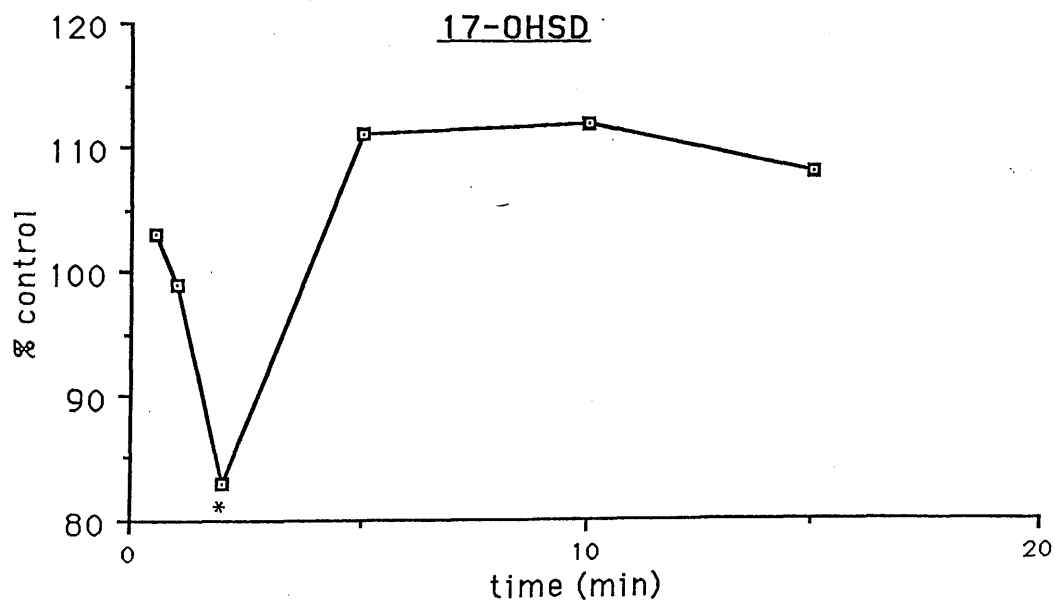
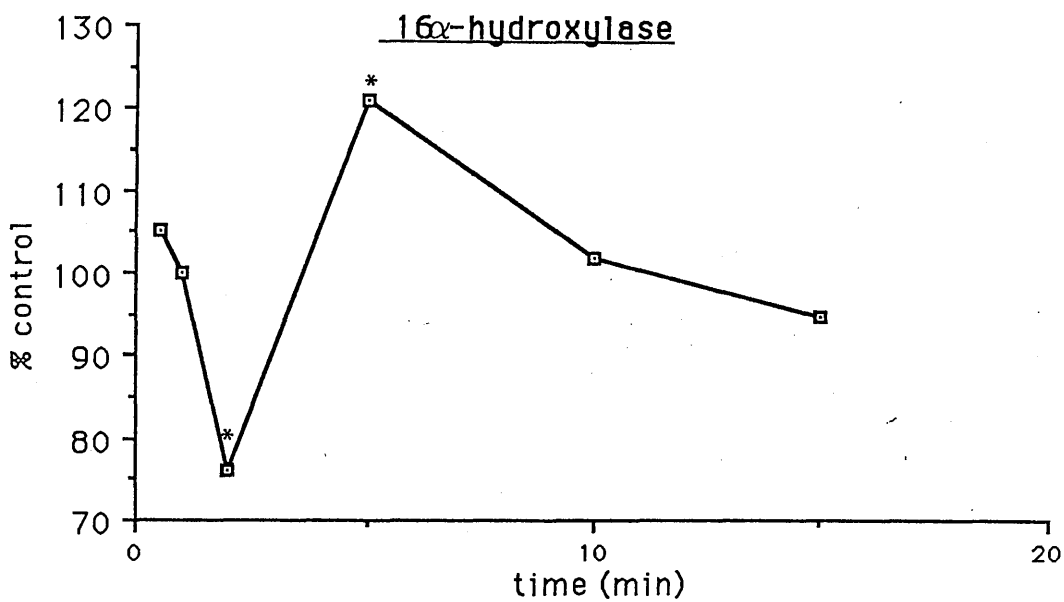
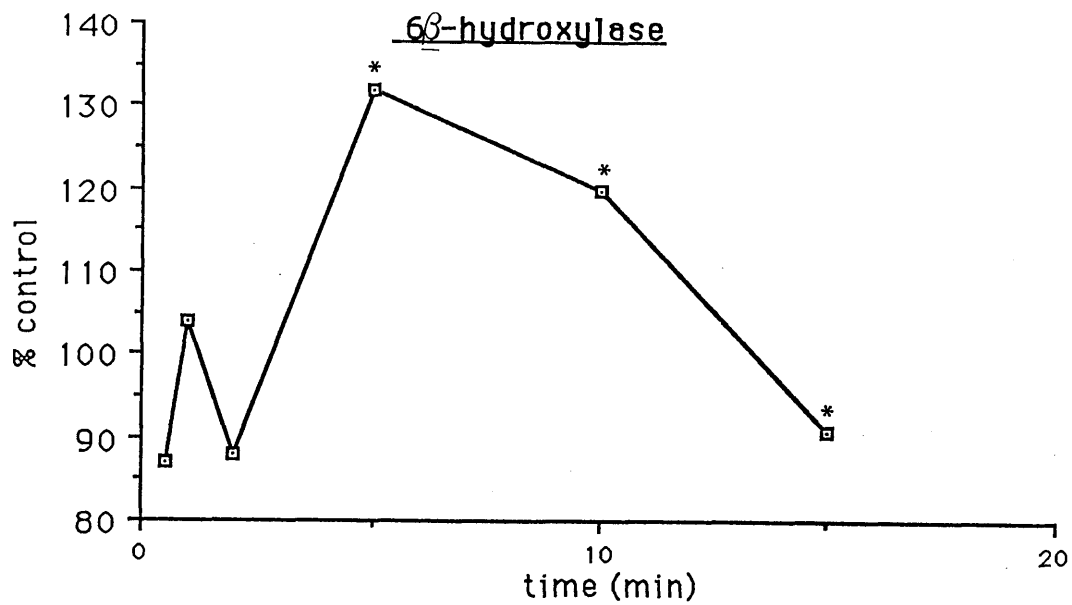


TABLE 30 : Time course of the production of the INSULIN MEDIATOR A) 1:2 dilution and B) 1:1 dilution upon treatment of hepatocytes with 10^{-9} M INSULIN for 0.5 to 15 minutes on 7 α -, 6 β -, and 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

A)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
0.5C		21 \pm 1	24 \pm 1	37 \pm 2	67 \pm 3	86 \pm 7
0.5IM		23 \pm 3	26 \pm 1*	41 \pm 4	67 \pm 4	74 \pm 4*
1.0C		20 \pm 1	22 \pm 2	50 \pm 4	67 \pm 3	72 \pm 8
1.0IM		21 \pm 4	22 \pm 1	37 \pm 6*	58 \pm 7*	69 \pm 8
2.0C		19 \pm 1	22 \pm 1	46 \pm 3	62 \pm 6	86 \pm 7
2.0IM		20 \pm 1	24 \pm 2	36 \pm 4*	52 \pm 9	76 \pm 8
5.0C		20 \pm 1	23 \pm 1	40 \pm 3	56 \pm 4	80 \pm 6
5.0IM		24 \pm 4	30 \pm 4*	49 \pm 7*	76 \pm 6*	118 \pm 11*
10.0C		23 \pm 1	28 \pm 2	64 \pm 6	78 \pm 4	96 \pm 11
10.0IM		23 \pm 1	27 \pm 1	52 \pm 8*	70 \pm 6	94 \pm 12
15.0C		24 \pm 2	32 \pm 4	52 \pm 2	76 \pm 3	96 \pm 2
15.0IM		24 \pm 1	30 \pm 1	59 \pm 4*	80 \pm 9	102 \pm 10

B)		Enzyme activities (pmoles/minute/million cells)				
TIME(min)		7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
0.5C		23 \pm 1	30 \pm 5	42 \pm 5	86 \pm 8	90 \pm 9
0.5IM		22 \pm 1	26 \pm 2	44 \pm 6	89 \pm 11	83 \pm 10
1.0C		22 \pm 1	27 \pm 3	49 \pm 3	75 \pm 9	116 \pm 14
1.0IM		21 \pm 1	28 \pm 3	49 \pm 6	74 \pm 8	92 \pm 5*
2.0C		29 \pm 3	32 \pm 3	54 \pm 2	96 \pm 4	114 \pm 17
2.0IM		26 \pm 1	28 \pm 3	41 \pm 4*	80 \pm 2*	94 \pm 2*
5.0C		19 \pm 1	22 \pm 1	42 \pm 5	72 \pm 8	104 \pm 11
5.0IM		24 \pm 2*	29 \pm 3*	51 \pm 1*	80 \pm 6	108 \pm 5
10.0C		20 \pm 3	28 \pm 4	52 \pm 6	76 \pm 8	102 \pm 8
10.0IM		23 \pm 3	34 \pm 1*	53 \pm 1	85 \pm 4	115 \pm 8*
15.0C		28 \pm 4	32 \pm 1	60 \pm 8	68 \pm 8	101 \pm 14
15.0IM		27 \pm 3	29 \pm 1*	53 \pm 8	65 \pm 1	106 \pm 14

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to control values.

minutes appeared to optimally stimulate steroid metabolism. Subsequent insulin mediator extracts were therefore prepared by incubation with insulin for 5 minutes.

3.6.5 TIME COURSE OF INSULIN MEDIATOR ACTION

Insulin mediator was prepared as detailed in section 2.8.2 and incubated with hepatocytes for 0 to 60 minutes in order to determine the time course of action (Table 31).

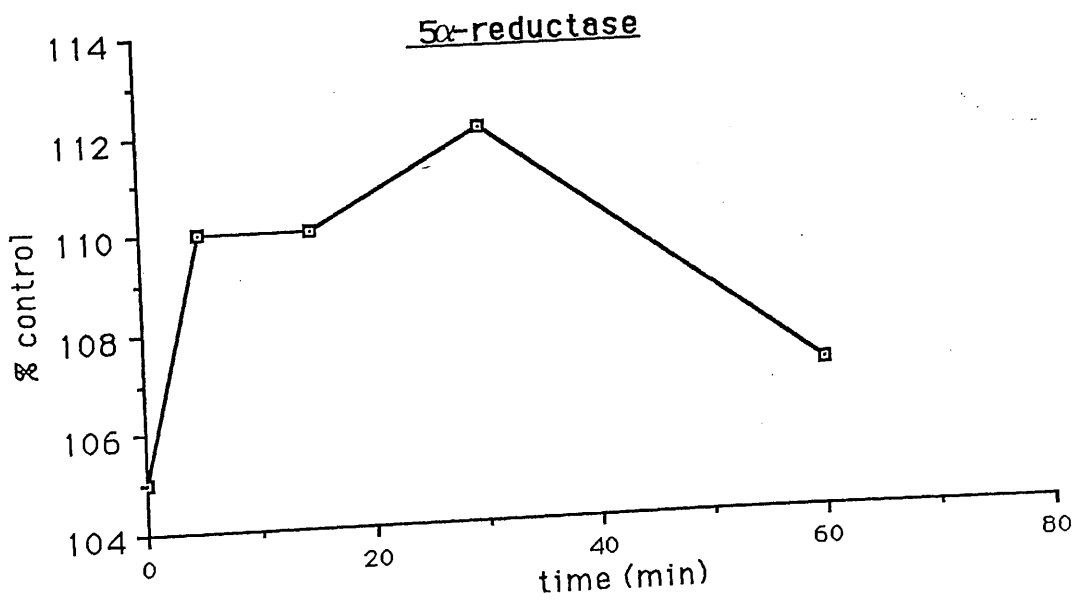
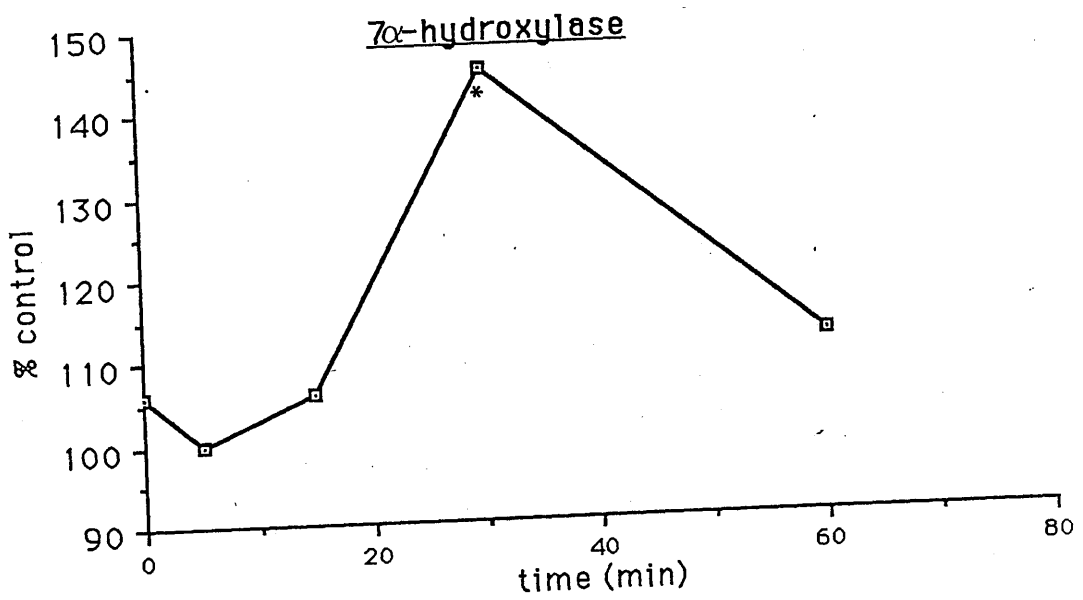
Figure 52A shows that 7 α -hydroxylase activity is maximal after 30 minutes of incubation, and remains unchanged at other time periods. 5 α -reductase, 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase activities (Figures 52B, 52C, 52D and 52E respectively) are gradually increased over the incubation period, reaching maxima at either 15 or 30 minutes of incubation.

Future incubations with the insulin mediator extract were therefore carried out at 30 minutes, since not all effects were apparent early in the time course.

3.6.6 DOSE-DEPENDENT EFFECTS OF 30 MINUTE INCUBATION OF HEPATOCYTES WITH VARIOUS DILUTIONS OF INSULIN MEDIATOR

Hepatocytes were preincubated for 30 minutes with various dilutions of the insulin mediator extract (Table 32). As is illustrated in Figure 53, enzyme activity was significantly stimulated upon incubation with both 1:2 and 1:1 dilutions of the insulin mediator extract. This

FIGURE 52 : Time course of the effects of preincubation of hepatocytes with 1:1 INSULIN MEDIATOR extract on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 31



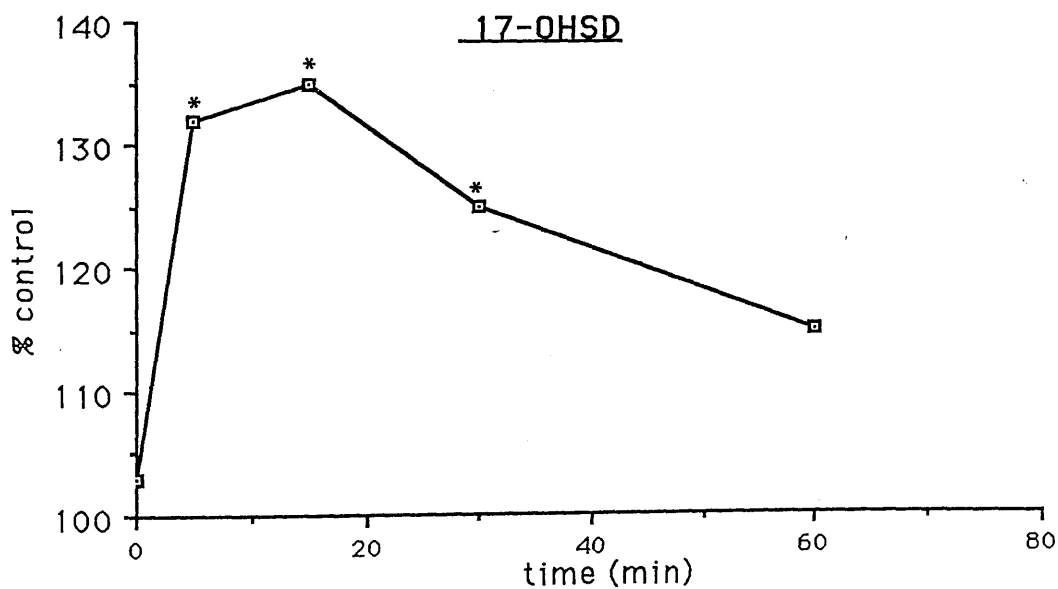
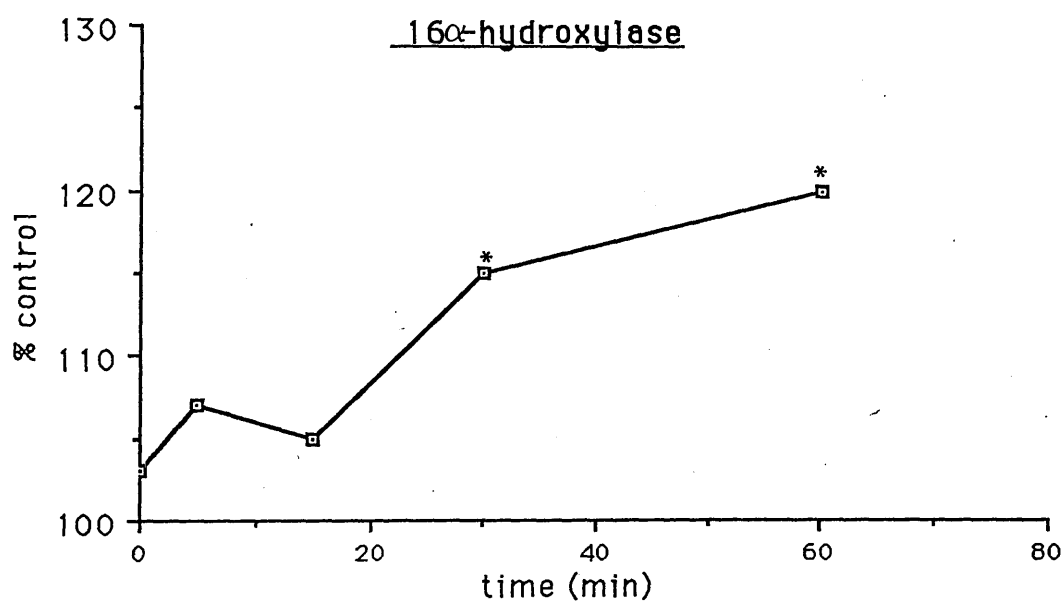
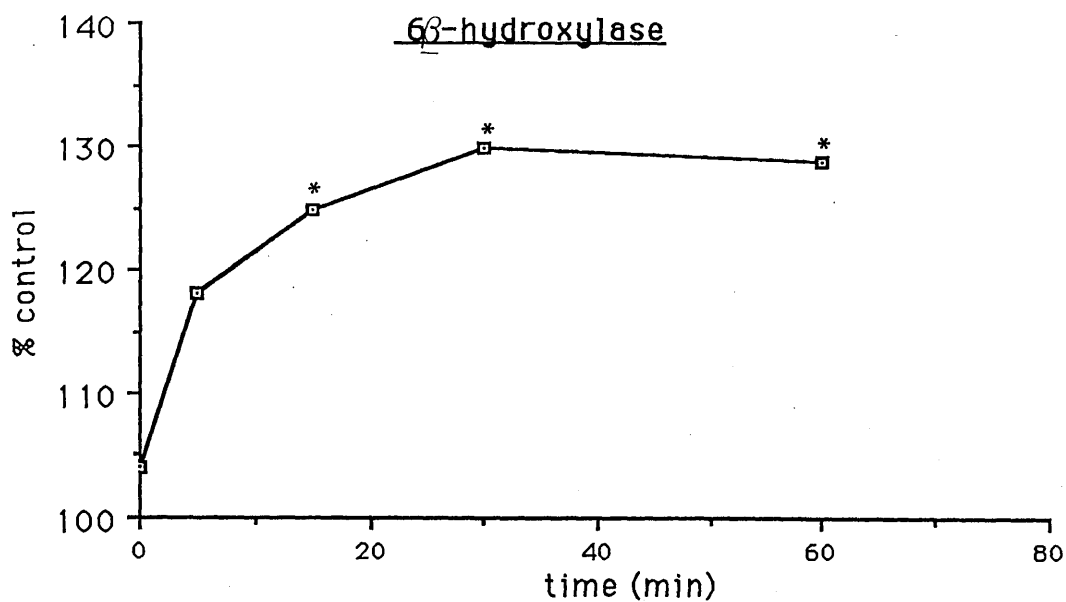
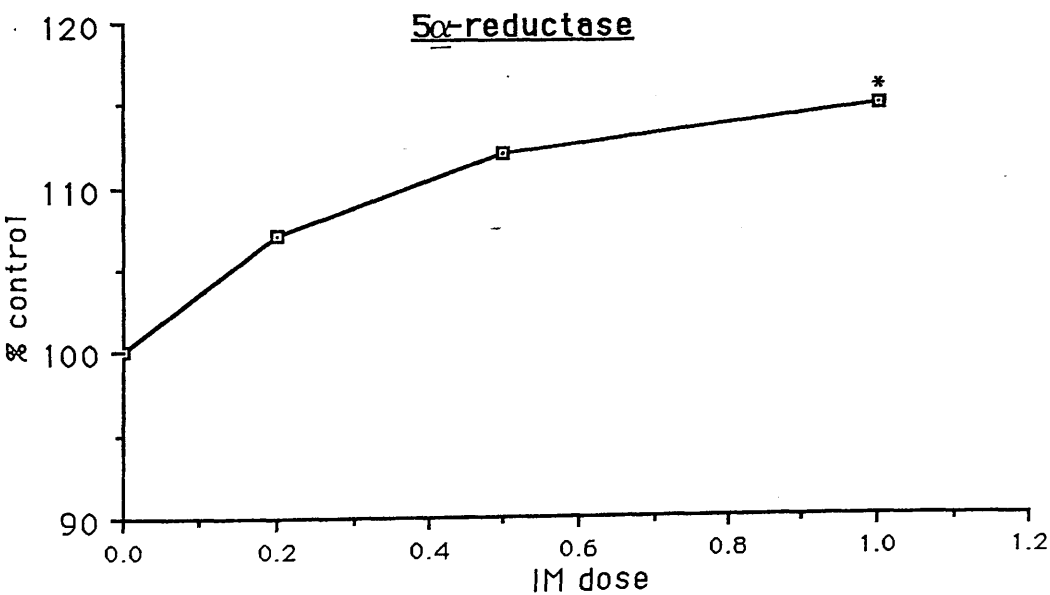
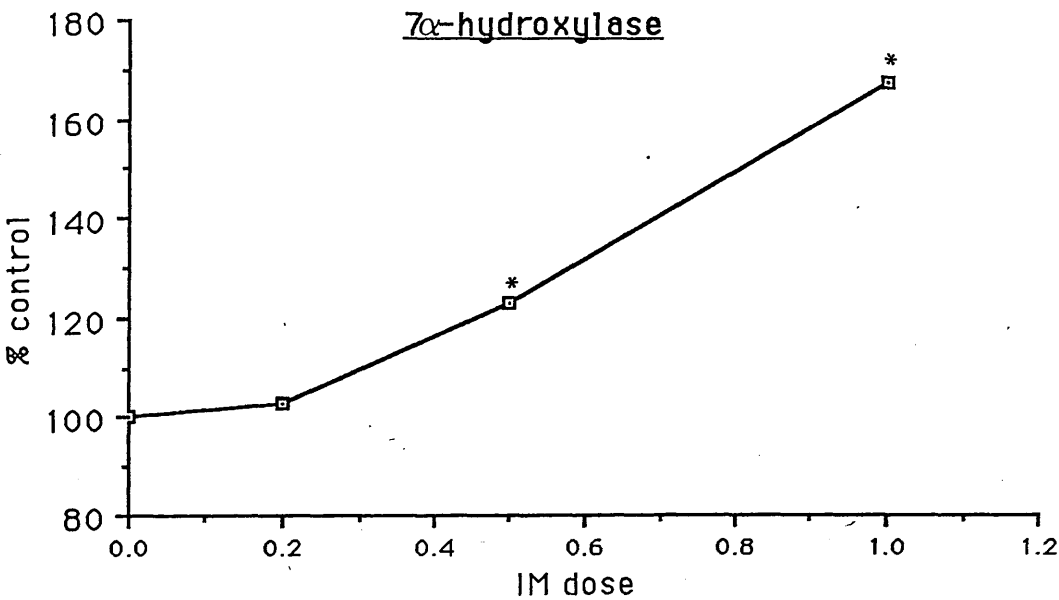


TABLE 31 : Time course of the effects of preincubation of hepatocytes with 1:1 INSULIN MEDIATOR extract on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

Enzyme activities (pmoles/minute/million cells)					
<u>TIME(min)</u>	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
control	20 \pm 1	22 \pm 1	36 \pm 1	78 \pm 1	80 \pm 2
0	21 \pm 1	23 \pm 1	37 \pm 2	80 \pm 4	84 \pm 3
5	20 \pm 1	26 \pm 1	39 \pm 2	103 \pm 4*	88 \pm 4
15	21 \pm 1	28 \pm 2*	38 \pm 2	105 \pm 6*	88 \pm 5
30	29 \pm 2*	29 \pm 1*	41 \pm 2*	98 \pm 7*	90 \pm 14
60	22 \pm 1	28 \pm 2*	43 \pm 1*	90 \pm 6	86 \pm 5

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to control.

TABLE 53 : Dose-dependent effects of 30 minute preincubation of hepatocytes with 1:5, 1:2 and 1:1 dilutions of the INSULIN MEDIATOR extract on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast = P < 0.05$ and $N = 3$. Absolute data is given in Table 32



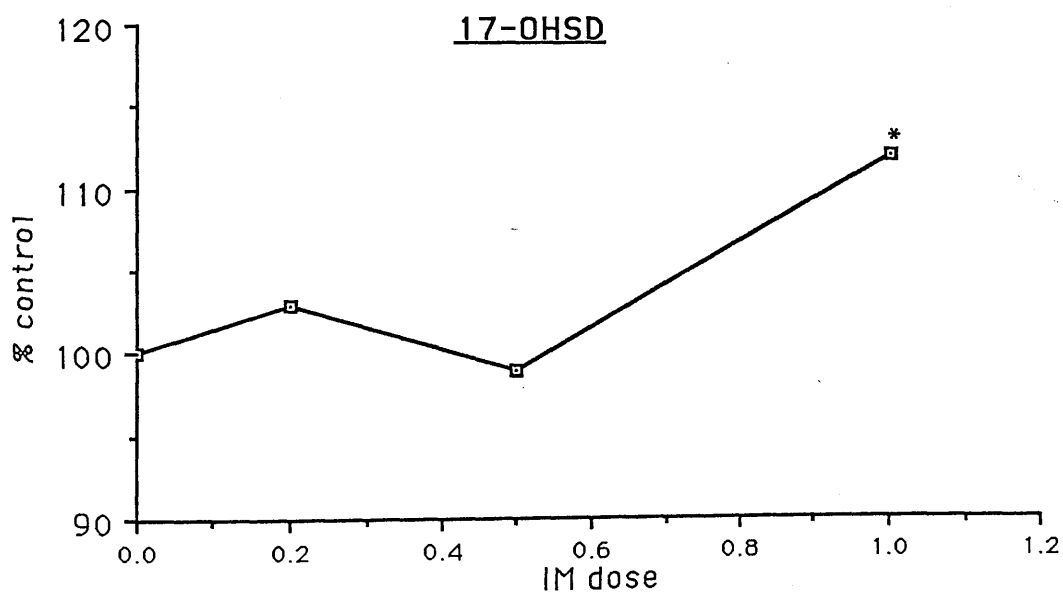
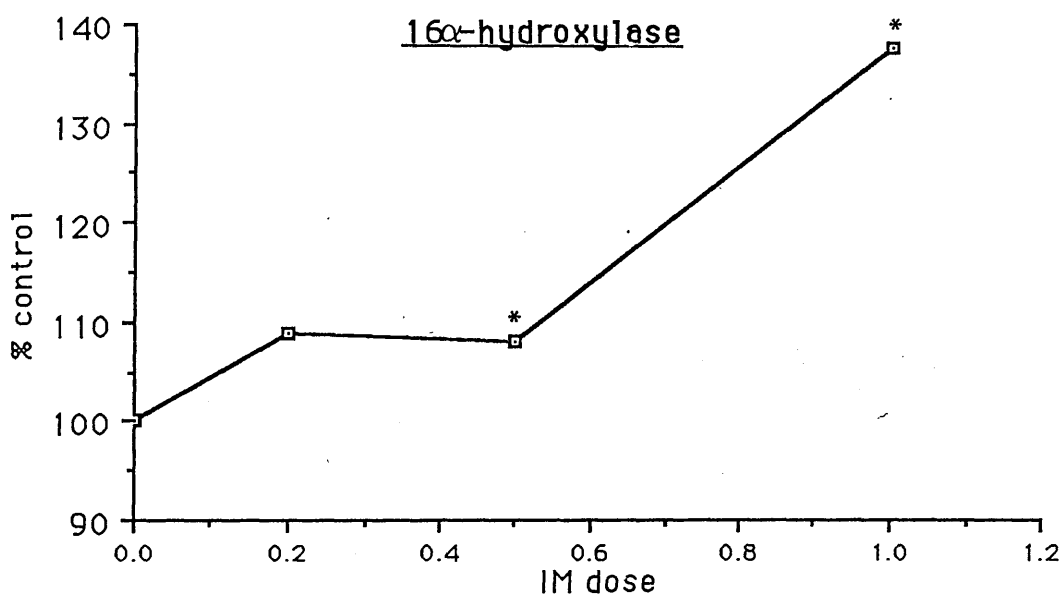
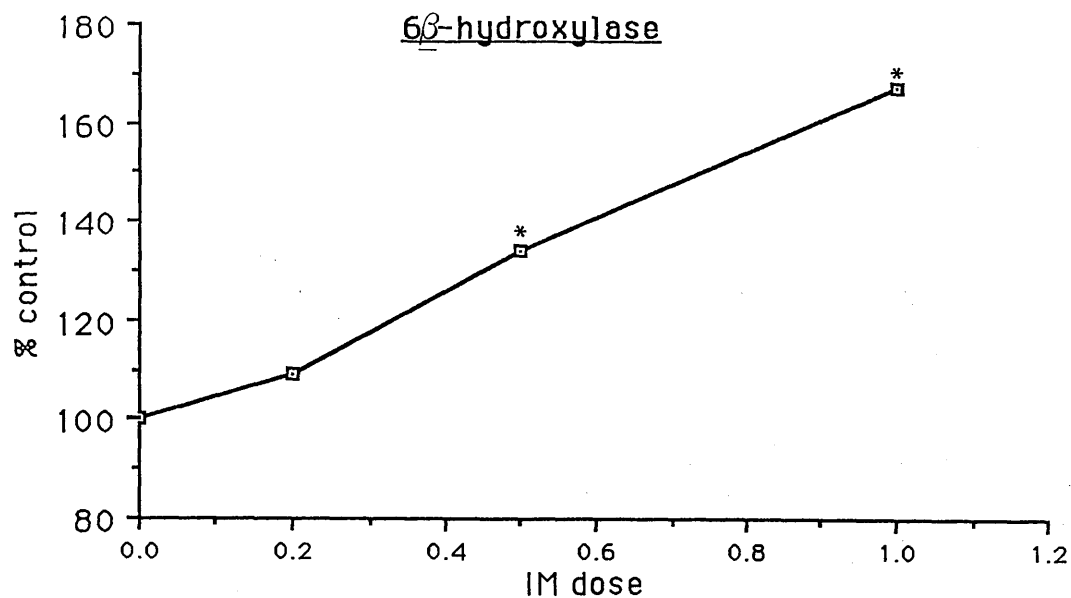


TABLE 32 : Dose-dependent effects of 30 minute preincubation of hepatocytes with 1:5, 1:2 and 1:1 dilutions of INSULIN MEDIATOR extract on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

<u>Enzyme activities (pmoles/minute/million cells)</u>					
<u>TREATMENT</u>	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
Control (A)	36 \pm 3	44 \pm 1	96 \pm 1	114 \pm 9	127 \pm 11
1:5 (B)	37 \pm 3	47 \pm 8	104 \pm 19	118 \pm 11	135 \pm 8
1:2 (C)	44 \pm 3*	58 \pm 1*	104 \pm 1*	114 \pm 11	141 \pm 15
1:1 (D)	60 \pm 3*	73 \pm 1*	132 \pm 3*	128 \pm 5*	146 \pm 2*

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to control.

Duncan's Multiple Range Test :

7 α -OHase	<u>A</u>	<u>B</u>	C	D
6 β -OHase	<u>A</u>	<u>B</u>	C	D
16 α -OHase	<u>A</u>	<u>B</u>	C	D
17-OHSD	<u>A</u>	<u>C</u>	<u>B</u>	D
5 α -red	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>

dose-dependent increase in enzyme activity was better illustrated with the P-450-dependent enzymes, 7 α -, 6 β -and 16 α -hydroxylases, whereas the P-450-independent enzymes, 17-oxosteroid oxidoreductase and 5 α -reductase, appeared relatively unresponsive to increasing doses of the insulin mediator extract.

3.6.7 EFFECT OF 30 MINUTE INCUBATION OF HEPATOCYTES
WITH INSULIN MEDIATOR AND ITS INTERACTION WITH 10⁻⁹M
GROWTH HORMONE

As is illustrated in Figure 54, incubation of hepatocytes with both insulin mediator and 10⁻⁹M growth hormone resulted in a significant decrease in enzyme activity (Table 33). A significant interaction was not, however, observed with 5 α -reductase activity upon incubation with growth hormone and insulin mediator, possibly since 5 α -reductase was not stimulated by the insulin mediator itself.

Thus the insulin mediator appears to interact with growth hormone in a similar manner to that observed with insulin and growth hormone before (section 3.2).

FIGURE 54 : Effects of 30 minute preincubation of hepatocytes with 1:1 INSULIN MEDIATOR extract in the absence and presence of 10^{-9} M GROWTH HORMONE on 7 α -hydroxylase, 5 α -reductase, 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 33

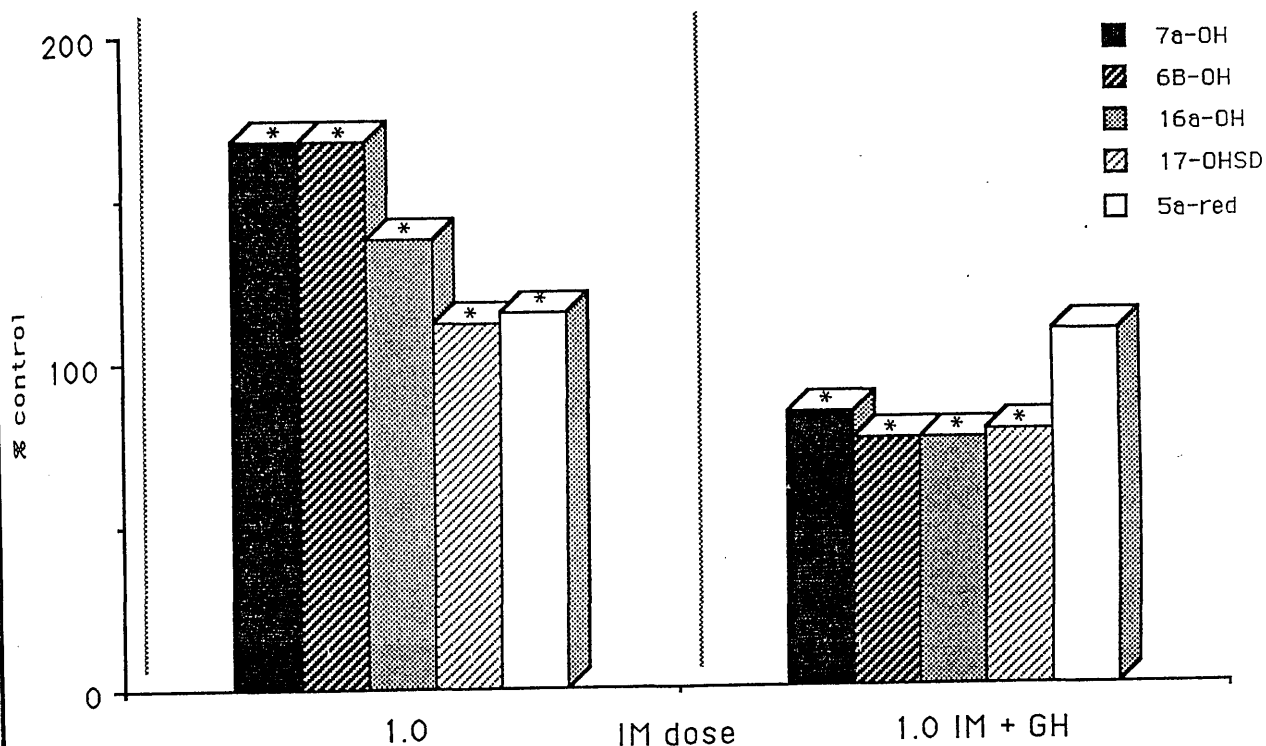


TABLE 33 : Effects of 30 minute preincubation of hepatocytes with 1:1 INSULIN MEDIATOR extract in the absence and presence of 10^{-9} M GROWTH HORMONE on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

TREATMENT	Enzyme activities (pmoles/minute/million cells)				
	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
Control	36 \pm 3	44 \pm 1	96 \pm 1	114 \pm 9	127 \pm 11
IM	60 \pm 3*	73 \pm 1*	132 \pm 3*	128 \pm 5*	146 \pm 2*
Control	49 \pm 4	69 \pm 4	99 \pm 8	126 \pm 4	130 \pm 1
IM+GH	41 \pm 2*	53 \pm 7*	75 \pm 5*	98 \pm 11*	141 \pm 13

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to relevant control values.

3.6.8 DOSE-DEPENDENT EFFECTS OF 30 MINUTE INCUBATION OF
LIVER MICROSOMES WITH VARIOUS DILUTIONS OF INSULIN
MEDIATOR

Incubation of liver microsomes with various doses of the insulin mediator extract exhibited dose-dependent effects on enzyme activity (Table 34). In all cases, enzyme activity was maximal at the lower concentrations of insulin mediator tested (Figure 55), and a reduction in enzyme activity was observed upon incubation with the undiluted insulin mediator extract.

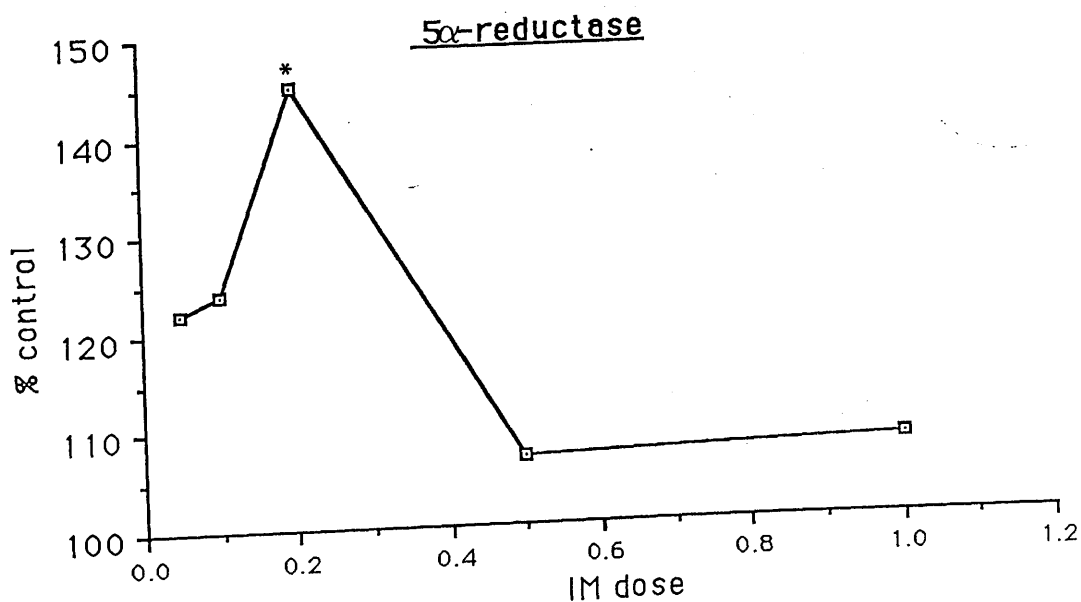
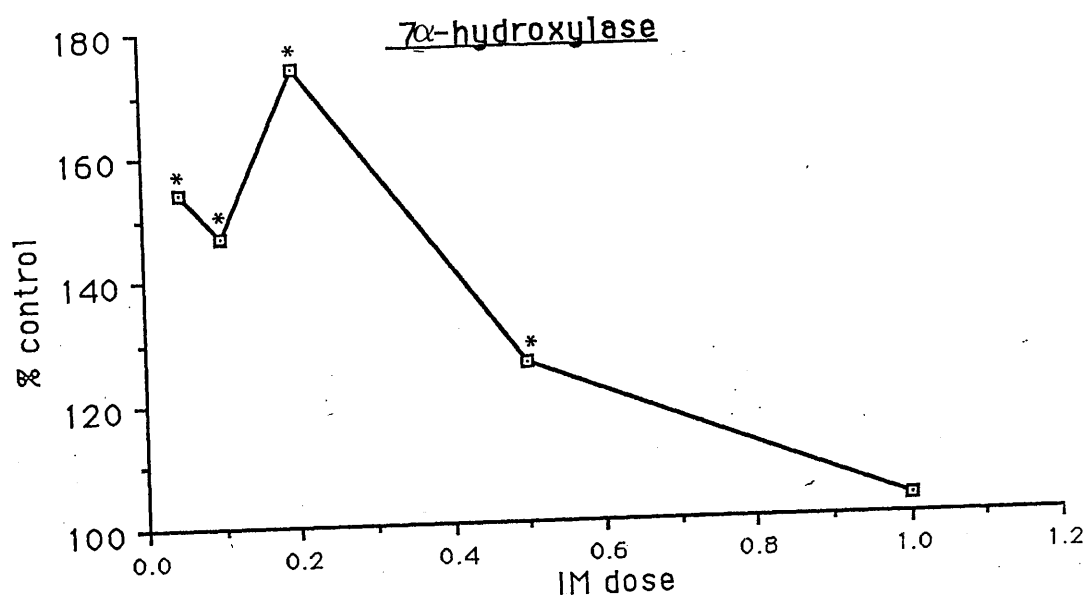
7 α -hydroxylase and 5 α -reductase activities (Figures 55A and 55B respectively) showed maximal stimulation with the 1:5 dilution of the original insulin mediator extract, and enzyme activity was restored to basal levels using the undiluted extract.

The male-specific enzyme activities all showed maximal stimulation with the 1:20 dilution of the insulin mediator extract (Figures 55C, 55D and 55E), again returning to basal levels with the undiluted extract. The P-450-independent 17-oxosteroid oxidoreductase activity (Figure 55E) appeared to be relatively unaffected in this case, as was seen in 3.6.7 above.

It therefore appears that the cell-free microsomal system is more responsive to the insulin mediator since maximum effects were exhibited at relatively low doses as compared to the situation with intact hepatocytes (see 3.6.7 above), and that the male-specific enzymes 6 β -hydroxylase and 16 α -hydroxylase are more sensitive to the insulin

FIGURE 55 : Dose-dependent effects of 30 minute preincubation of rat liver microsomes with 1:20, 1:10, 1:5, 1:2 and 1:1 dilutions of the INSULIN MEDIATOR extract on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $\ast = P < 0.05$ and $N = 3$. Absolute data is given in Table 34



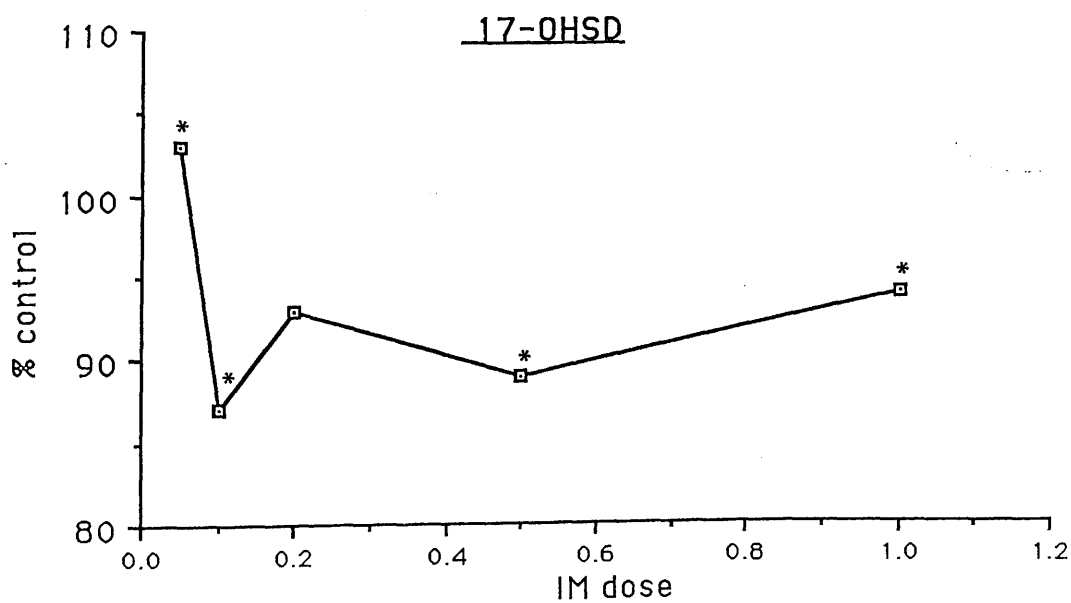
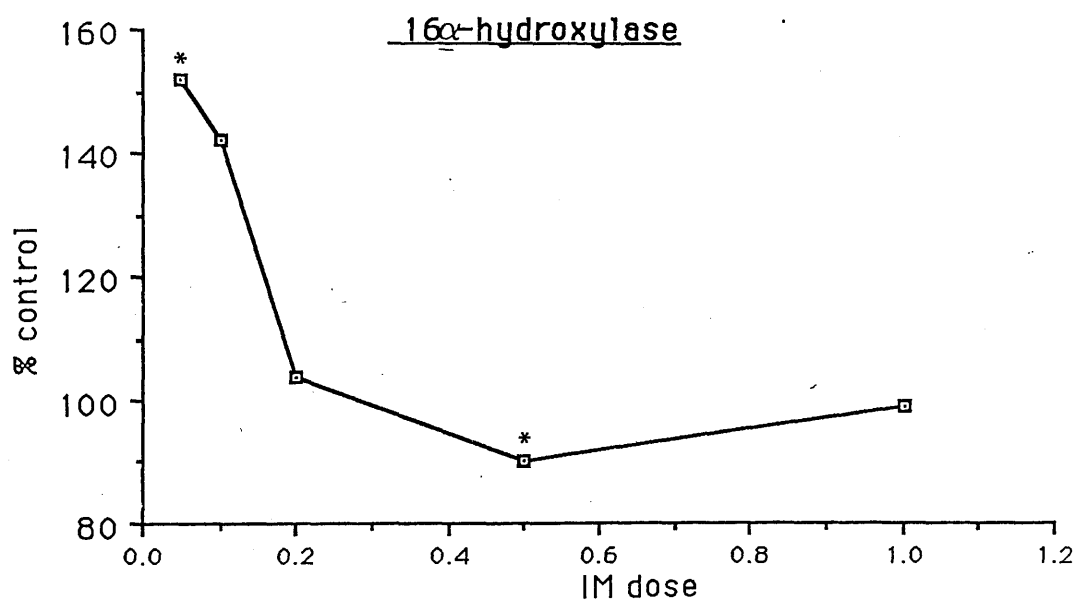
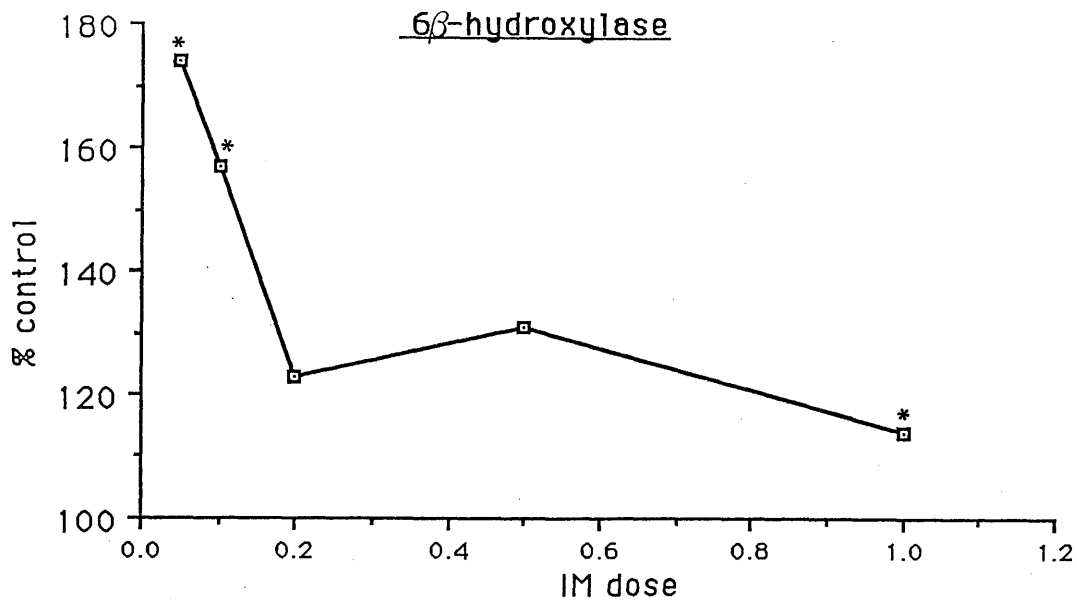


TABLE 34 : Dose-dependent effects of 30 minute preincubation of rat liver microsomes with 1:20, 1:10, 1:5, 1:2 and 1:1 dilutions of INSULIN MEDIATOR extract on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

Enzyme activities (pmoles/minute/mg protein)					
TREATMENT	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
Control (A)	38 \pm 2	58 \pm 4	87 \pm 5	134 \pm 5	150 \pm 12
1:20 (B)	74 \pm 2*	92 \pm 2*	126 \pm 12*	195 \pm 10*	161 \pm 8
1:10 (C)	48 \pm 4*	90 \pm 2*	90 \pm 2	160 \pm 12*	151 \pm 1
1:5 (D)	66 \pm 1*	62 \pm 2	104 \pm 4	141 \pm 6	206 \pm 6*
1:2 (E)	50 \pm 5*	61 \pm 5	94 \pm 2*	143 \pm 6*	158 \pm 4
1:1 (F)	39 \pm 4	42 \pm 2*	90 \pm 3	150 \pm 2*	158 \pm 1

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to control.

Duncan's Multiple Range Test :

7 α -OHase	<u>A</u>	<u>F</u>	<u>C</u>	<u>E</u>	D	B
6 β -OHase	F	<u>A</u>	<u>E</u>	<u>D</u>	<u>C</u>	<u>B</u>
16 α -OHase	<u>A</u>	<u>C</u>	<u>F</u>	<u>E</u>	D	B
17-OHSD	<u>A</u>	<u>D</u>	<u>E</u>	<u>F</u>	<u>C</u>	<u>B</u>
5 α -red	<u>A</u>	<u>C</u>	<u>E</u>	<u>F</u>	B	D

mediator than the female-specific enzymes 7 α -hydroxylase and 5 α -reductase, since a lower dose was required to obtain the same effect.

3.6.9 ELUTION PROFILE OF THE INSULIN MEDIATOR EXTRACT ON AG1x8 ION-EXCHANGE CHROMATOGRAPHY

Insulin mediator was applied undiluted to an AG1x8 ion-exchange resin and sequentially eluted with increasing concentrations of ammonium formate, as detailed in section 2.8.1. The eluted fractions were then incubated with hepatocytes for 30 minutes in order to determine which fraction contained the insulin mediator. As is shown in Table 35 and in Figure 56, enzyme activity was significantly increased upon incubation with the 1.0M ammonium formate fraction 1, and to a greater extent with the fraction 2 eluate. Some residual activity also appeared to be present in the 1.0M formic acid eluate. Some significant increases in enzyme activity were observed with the 1mM and 0.2M eluates which may be attributed to the elution of nucleotides. Thus the insulin mediator activity appeared to be eluted mainly in the 1.0M ammonium formate fraction 2, and to a lesser extent in fraction 1. Remaining bound mediator activity was eluted in the 1.0M formic acid eluate. Fraction 2 was therefore used for further assay.

FIGURE 56 : Effects of 30 minute preincubation of hepatocytes with 1mM formic acid, 0.025M, 0.1M, 0.2M, 0.5M, 1.0M ammonium formate and 1M formic acid AG1x8 eluates and of the unpurified INSULIN MEDIATOR extract on 7 α -hydroxylase, 5 α -reductase, 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where $\ast=P<0.05$ and $N=3$. Absolute data is given in Table 35

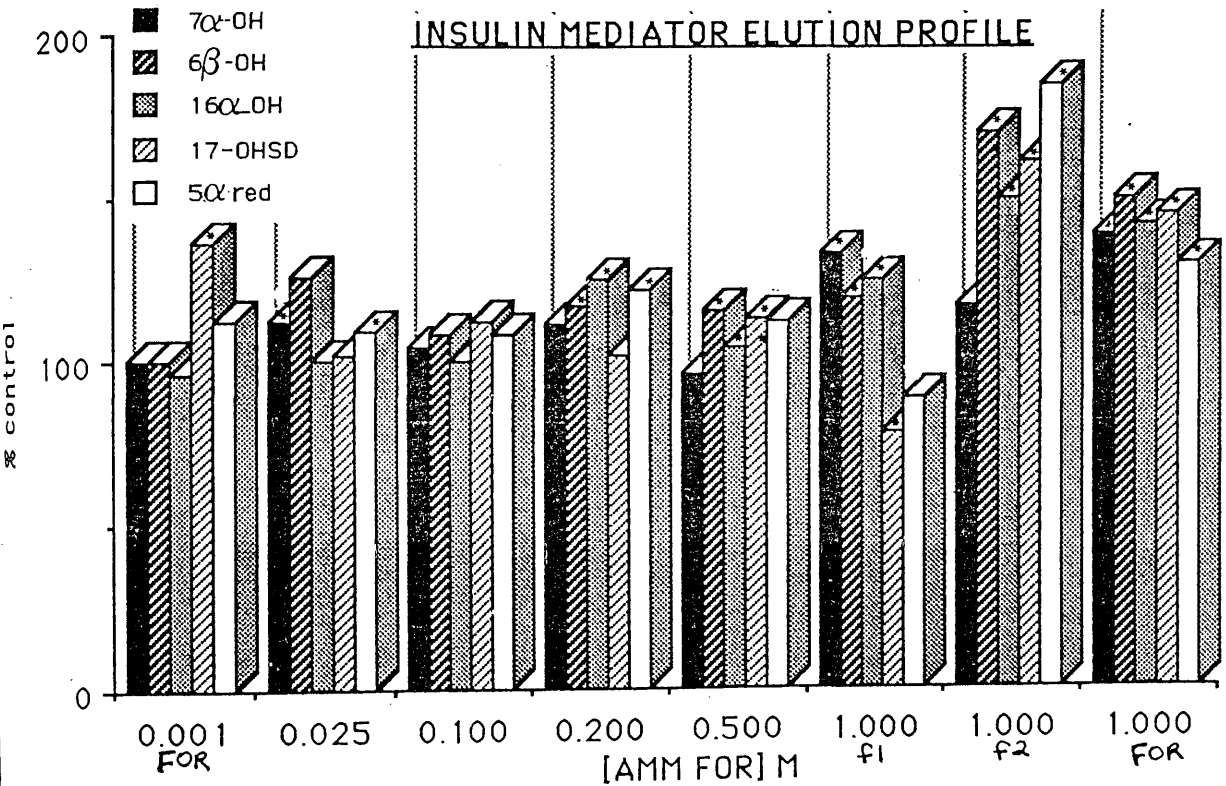


TABLE 35 : Effects of 30 minute preincubation of hepatocytes with 1mM formic acid (FOR) ; 0.025M, 0.1M, 0.2M, 0.5M, 1.0M (fraction 1), 1.0M (fraction 2) ammonium formate (AF) and 1M formic acid (FOR) AG1x8 eluates and of the unpurified INSULIN MEDIATOR extract on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

Enzyme activities (pmoles/minute/million cells)					
TREATMENT	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
control	24 \pm 2	34 \pm 3	78 \pm 6	90 \pm 9	57 \pm 9
1:1 IM	28 \pm 3	48 \pm 2*	81 \pm 11	120 \pm 1*	112 \pm 4*
control	24 \pm 1	36 \pm 3	50 \pm 2	44 \pm 4	68 \pm 6
1mM FOR	24 \pm 4	36 \pm 4	48 \pm 2	60 \pm 7*	76 \pm 9
control	25 \pm 1	27 \pm 3	72 \pm 1	82 \pm 3	98 \pm 1
0.025M AF	28 \pm 1*	34 \pm 7	72 \pm 1	84 \pm 1	107 \pm 1*
control	26 \pm 2	24 \pm 3	52 \pm 2	84 \pm 1	94 \pm 10
0.1M AF	27 \pm 7	26 \pm 1	52 \pm 1	94 \pm 10	102 \pm 1
control	28 \pm 3	36 \pm 2	60 \pm 2	97 \pm 2	94 \pm 6
0.2M AF	31 \pm 4	42 \pm 1*	75 \pm 4*	99 \pm 6	115 \pm 2*
control	26 \pm 2	26 \pm 1	46 \pm 1	70 \pm 1	86 \pm 10
0.5M AF	25 \pm 1	30 \pm 1*	48 \pm 1*	79 \pm 1*	96 \pm 1
control	27 \pm 2	26 \pm 1	48 \pm 2	86 \pm 5	118 \pm 7
1.0M AF, f1	36 \pm 5*	31 \pm 1*	60 \pm 1*	67 \pm 8*	105 \pm 1
control	24 \pm 5	20 \pm 1	48 \pm 1	62 \pm 1	96 \pm 2
1.0M AF, f2	28 \pm 3	34 \pm 4*	72 \pm 8*	100 \pm 4*	178 \pm 18*
control	23 \pm 2	22 \pm 3	45 \pm 2	62 \pm 7	100 \pm 6
1.0M FOR	32 \pm 4*	33 \pm 1*	64 \pm 1*	90 \pm 11*	130 \pm 13*

Results are expressed as MEAN \pm S.D. (N=3); *=P, 0.05 as compared to relevant control values.

3.6.10 DOSE-DEPENDENT EFFECTS OF 30 MINUTE
PREINCUBATION OF HEPATOCYTES WITH VARIOUS DILUTIONS OF
AG1x8-PURIFIED INSULIN MEDIATOR EXTRACT.

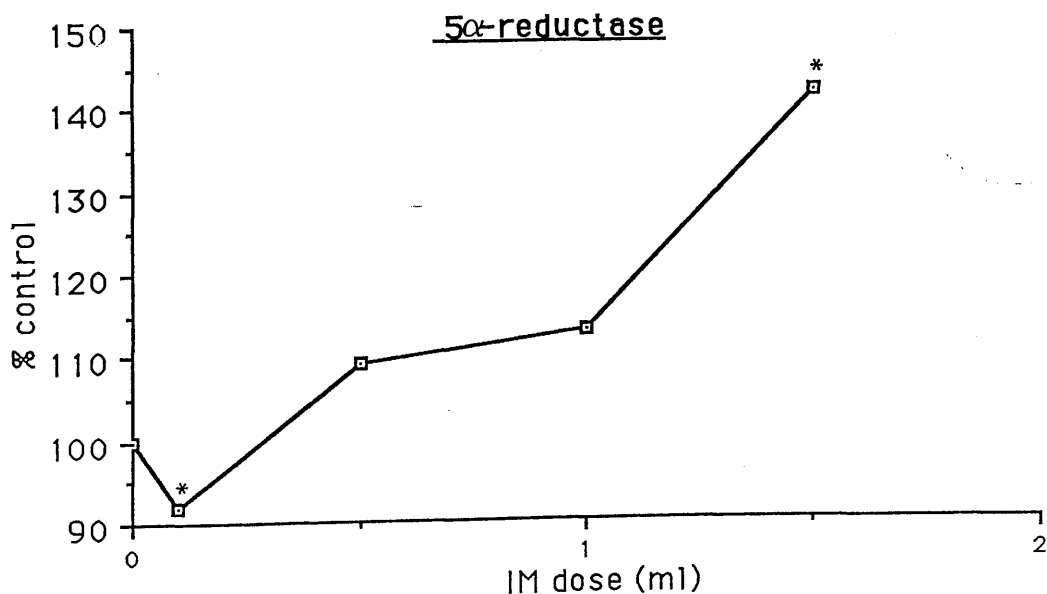
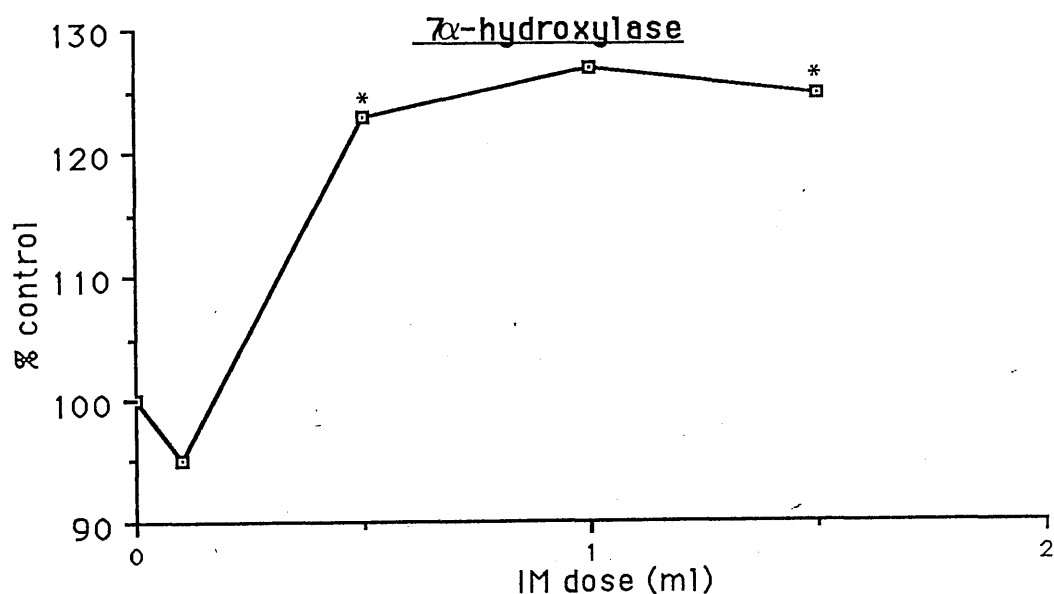
Table 36 shows the effects of incubation of hepatocytes with AG1x8-purified insulin mediator extract. As is illustrated in Figure 57, all activities remained unchanged upon incubation with a 1:10 dilution of the extract, and a dose-dependent increase in enzyme activity was exhibited thereafter with more concentrated extract. Enzyme activity reached a maximum with the highest dose of insulin mediator assayed (3:2), although this was not the case with 6 β -hydroxylase activity (Figure 57C).

3.6.11 DOSE-DEPENDENT EFFECTS OF 30 MINUTE
PREINCUBATION OF LIVER MICROSOMES WITH AG1x8-PURIFIED
INSULIN MEDIATOR

Table 37 shows the effects of incubation of liver microsomes with varying doses of AG1x8-purified insulin mediator. As is illustrated in Figure 58, enzyme activity exhibited maxima using the undiluted fraction. Further, P-450-dependent enzyme activities 7 α -hydroxylase, 6 β -hydroxylase and 16 α -hydroxylase (Figures 58A, 58C and 58D respectively) were increased markedly with 1:5 and 1:2 dilutions of the purified extract, although a peak in enzyme activity was demonstrated with the undiluted extract. The P-450-independent activities 5 α -reductase and 17-oxosteroid oxidoreductase (Figures 58B and 58E respectively) however

FIGURE 57 : Dose-dependent effects of 30 minute preincubation of hepatocytes with 1:10, 1:2, 1:1 and 3:2 dilutions of the AG1x8-purified INSULIN MEDIATOR extract on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 36



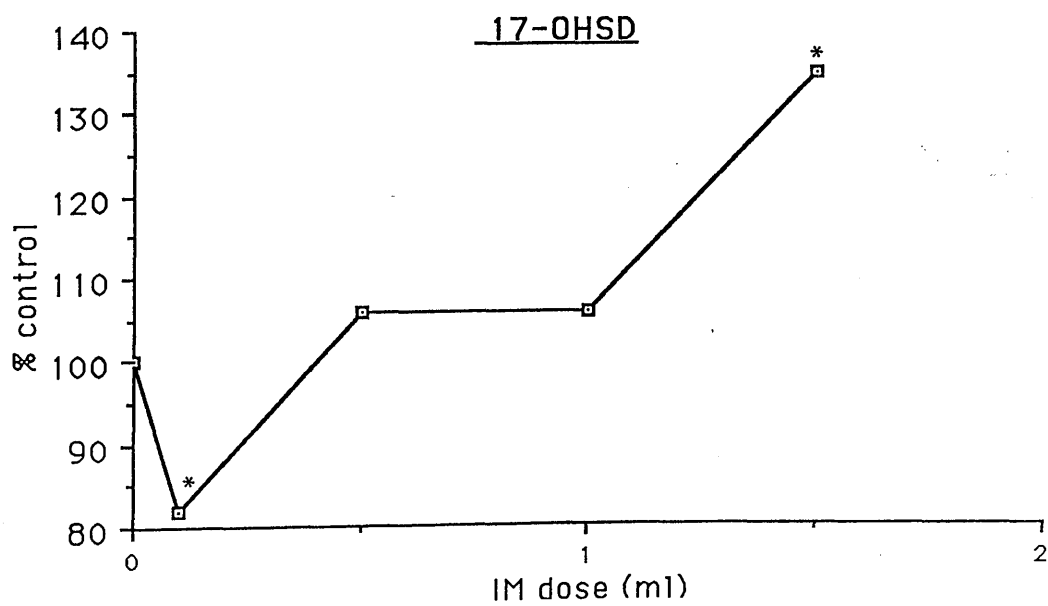
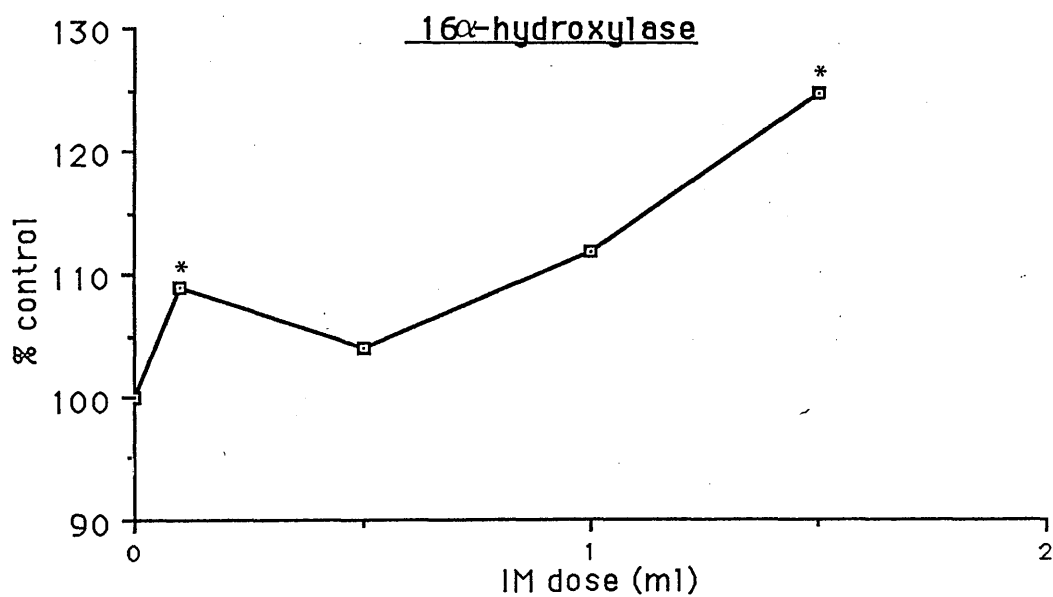
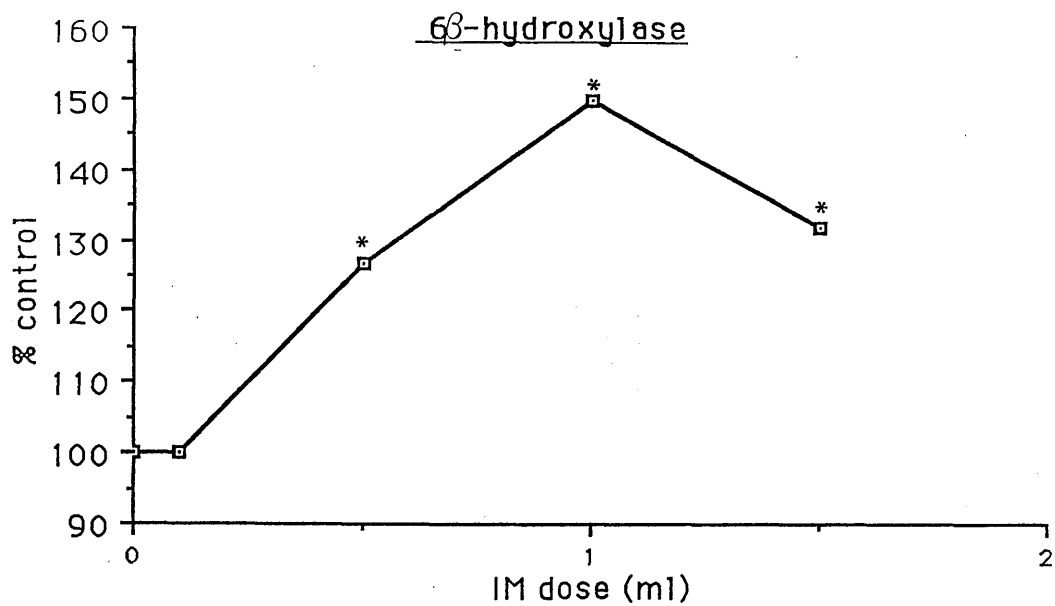
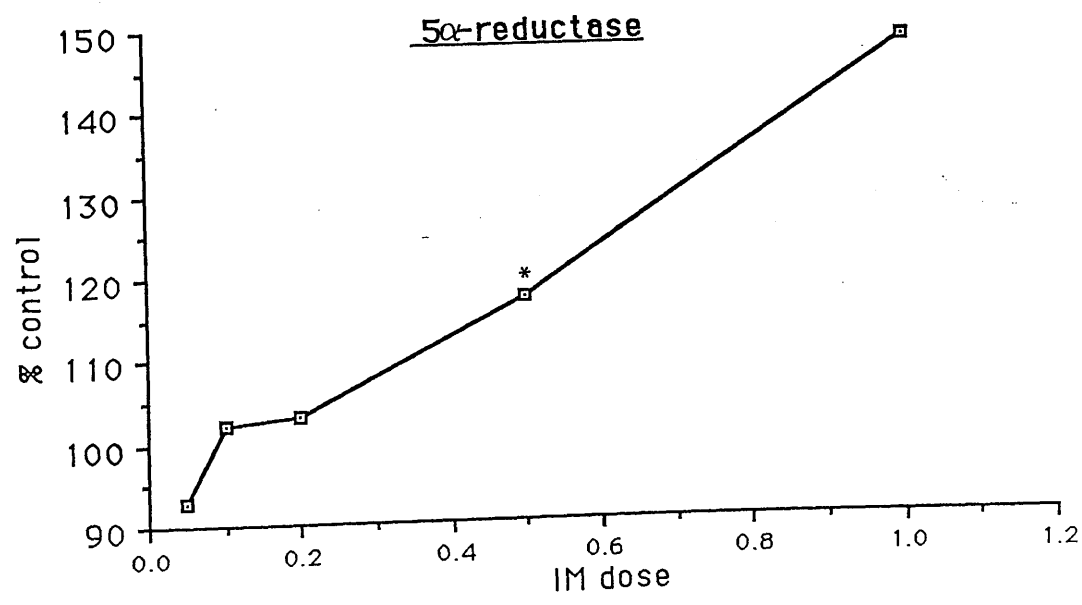
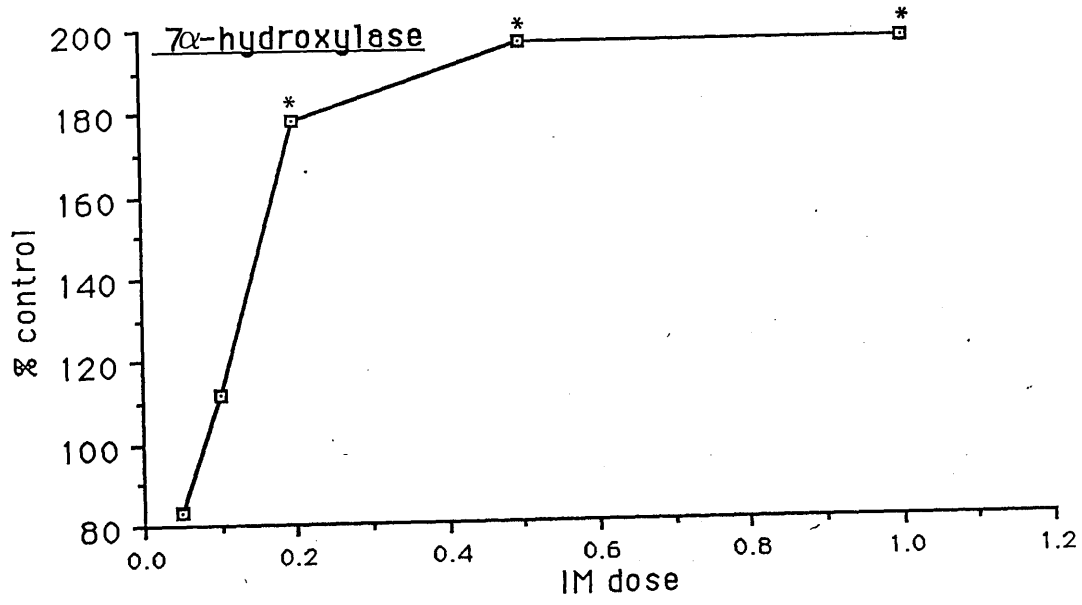


TABLE 36 : Dose-dependent effects of 30 minute preincubation of hepatocytes with 1:10, 1:2, 1:1 and 3:2 dilutions of the AG1x8-purified INSULIN MEDIATOR extract on 7 α -, 6 β - and 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase.

<u>Enzyme activities (pmoles/minute/million cells)</u>					
<u>IM dilution</u>	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
control	22 \pm 3	28 \pm 2	46 \pm 1	44 \pm 1	100 \pm 1
1:10	21 \pm 1	28 \pm 6	50 \pm 2*	36 \pm 1*	92 \pm 2*
control	26 \pm 1	18 \pm 1	28 \pm 1	64 \pm 6	86 \pm 9
1:2	32 \pm 3*	23 \pm 4*	29 \pm 1	68 \pm 3	94 \pm 8
control	22 \pm 2	20 \pm 1	32 \pm 2	36 \pm 3	94 \pm 5
1:1	28 \pm 5	30 \pm 1*	36 \pm 4	38 \pm 1	106 \pm 11
control	19 \pm 2	25 \pm 3	29 \pm 2	50 \pm 7	92 \pm 4
3:2	24 \pm 3*	33 \pm 2*	36 \pm 3*	68 \pm 5*	131 \pm 12*

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to relevant control values.

FIGURE 58 : Dose-dependent effects of 30 minute preincubation of rat liver microsomes with 1:20, 1:10, 1:5, 1:2 and 1:1 dilutions of the AG1x8-purified INSULIN MEDIATOR extract on A) 7 α -hydroxylase, B) 5 α -reductase, C) 6 β -hydroxylase, D) 16 α -hydroxylase and E) 17-oxosteroid oxidoreductase activities. Results are expressed as percentages of relevant control values, where *=P<0.05 and N=3. Absolute data is given in Table 37



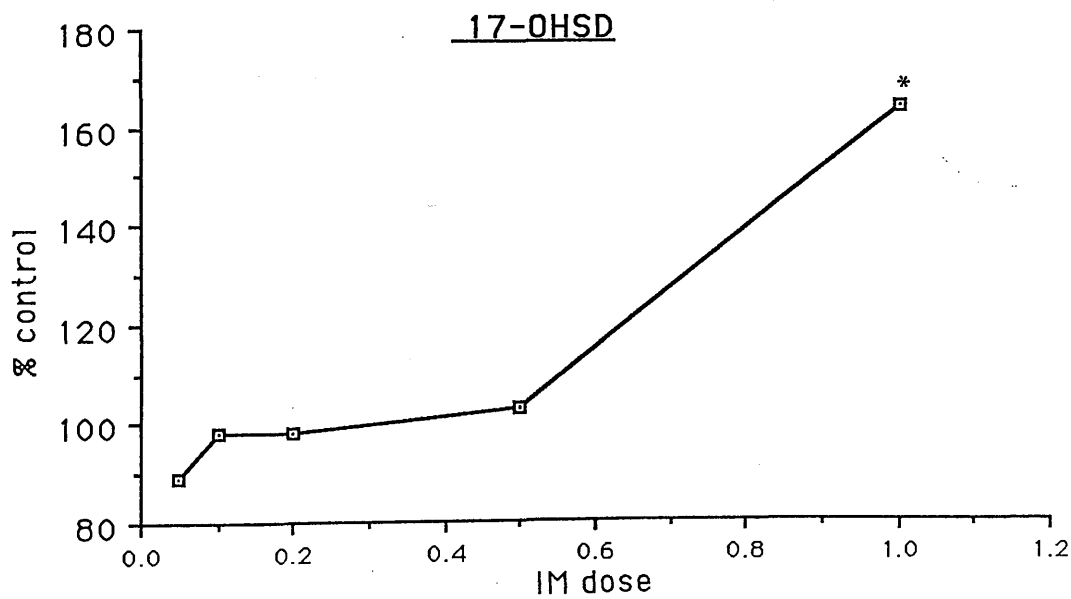
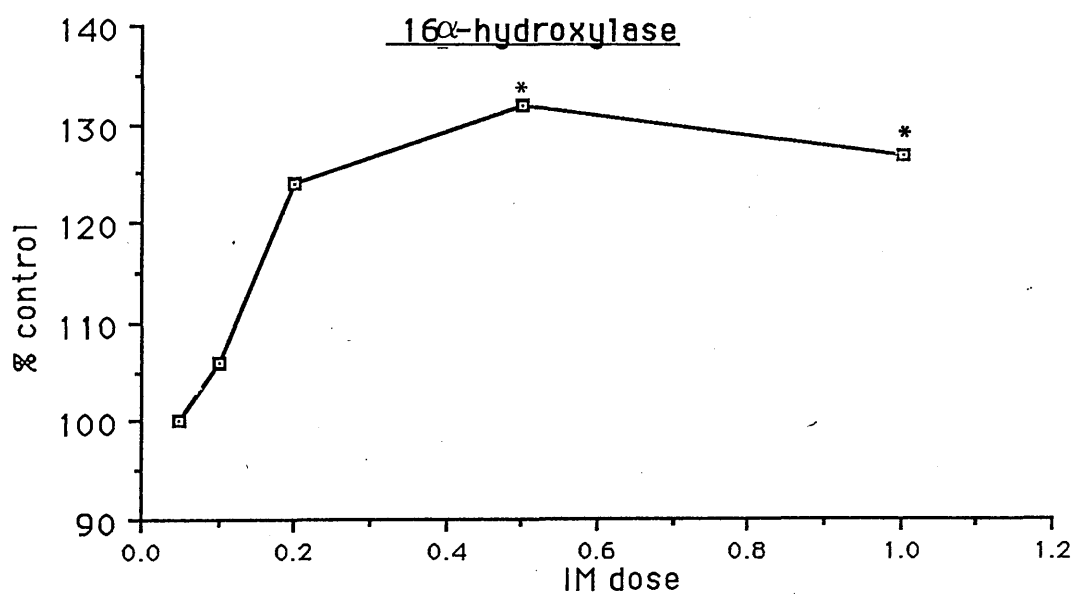
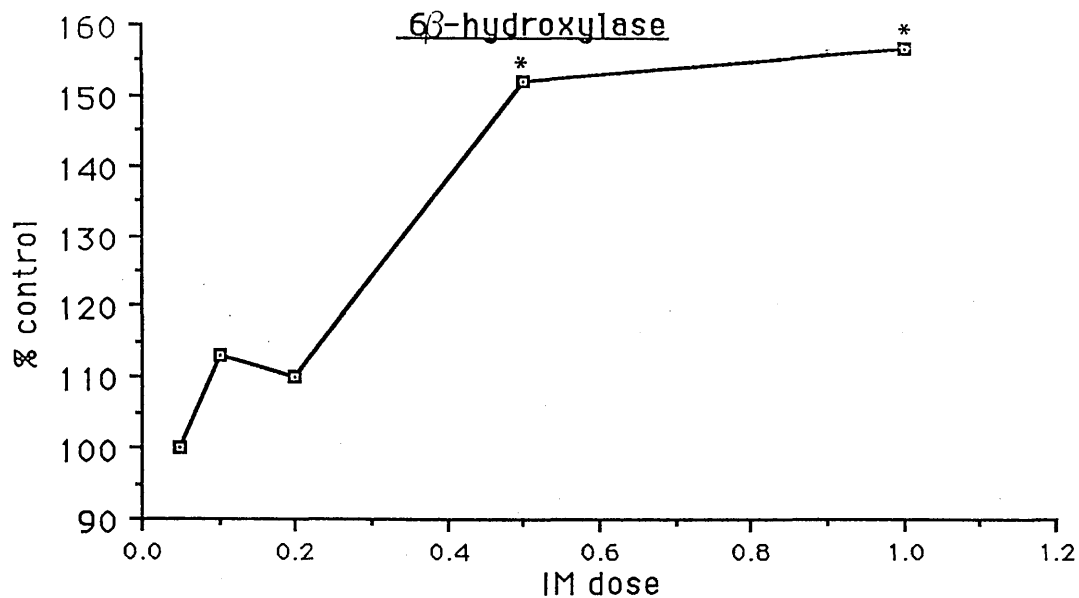


TABLE 37 : Dose-dependent effects of 30 minute preincubation of rat liver microsomes with 1:20, 1:10, 1:5, 1:2 and 1:1 dilutions of AG1x8-purified INSULIN MEDIATOR on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

Enzyme activities (pmoles/minute/mg protein)					
TREATMENT	7 α -OHase	6 β -OHase	16 α -OHase	17-OHSD	5 α -red
Control (A)	39 \pm 3	77 \pm 7	93 \pm 4	99 \pm 7	101 \pm 9
1:20 (B)	41 \pm 2	80 \pm 3	102 \pm 2	103 \pm 6	102 \pm 2
1:10 (C)	41 \pm 4	83 \pm 4	94 \pm 9	111 \pm 3	100 \pm 10
1:5 (D)	73 \pm 3*	72 \pm 3	91 \pm 4	114 \pm 4	107 \pm 2
1:2 (E)	72 \pm 2*	90 \pm 5*	98 \pm 1*	104 \pm 7	126 \pm 4*
1:1 (F)	66 \pm 3*	88 \pm 4*	114 \pm 6*	136 \pm 14*	162 \pm 15

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to control.

Duncan's Multiple Range Test :

7 α -OHase	<u>A</u>	<u>B</u>	<u>C</u>	<u>F</u>	<u>E</u>	<u>D</u>
6 β -OHase	<u>D</u>	<u>A</u>	<u>B</u>	<u>C</u>	<u>F</u>	<u>E</u>
16 α -OHase	<u>D</u>	<u>A</u>	<u>C</u>	<u>E</u>	<u>B</u>	<u>F</u>
17-OHSD	<u>A</u>	<u>B</u>	<u>E</u>	<u>C</u>	<u>D</u>	<u>F</u>
5 α -red	<u>C</u>	<u>A</u>	<u>B</u>	<u>D</u>	<u>E</u>	<u>F</u>

showed no marked stimulation of enzyme activity other than with the undiluted extract.

Thus the P-450-dependent enzymes appear to be more responsive to the insulin mediator extract. Enzyme activity was markedly increased with the more diluted original extract (see 3.6.8) as compared to the relatively higher doses of the purified extract required here. This may be due to the fact that all of the insulin mediator activity is not eluted in the fraction used here, but rather appears to be eluted in three separate fractions, as was shown in section 3.6.10.

3.6.12 EFFECTS OF 30 MINUTE INCUBATION OF LIVER MICROSOMES WITH AG1x8-PURIFIED INSULIN MEDIATOR AND ITS INTERACTION WITH 10^{-9} M GROWTH HORMONE

Rat liver microsomes were incubated with undiluted, AG1x8-purified insulin mediator and 10^{-9} M growth hormone for 30 minutes (Table 38). Growth hormone appeared to antagonise the stimulatory effects of the purified-mediator fraction (Figure 59).

3.6.13 CHROMATOGRAPHY OF THE INSULIN MEDIATOR EXTRACT

Insulin mediator extract, prepared as described in section 2.8.2, was subjected to acid-hydrolysis (section 2.11) and samples were spotted onto 20x20cm squares of Whatman No. 1 filter paper. 5% standard solutions of galactose, mannose, galactosamine, glucosamine and inositol,

FIGURE 59 : Effects of 30 minute preincubation of rat liver microsomes with 1:1 AG1x8-purified INSULIN MEDIATOR extract in the absence and presence of 10^{-9} M GROWTH HORMONE on 7 α -hydroxylase, 5 α -reductase, 6 β -hydroxylase, 16 α -hydroxylase and 17-oxosteroid oxidoreductase activities.

Results are expressed as percentages of relevant control values, where $*=P<0.05$ and $N=3$. Absolute data is given in Table 38

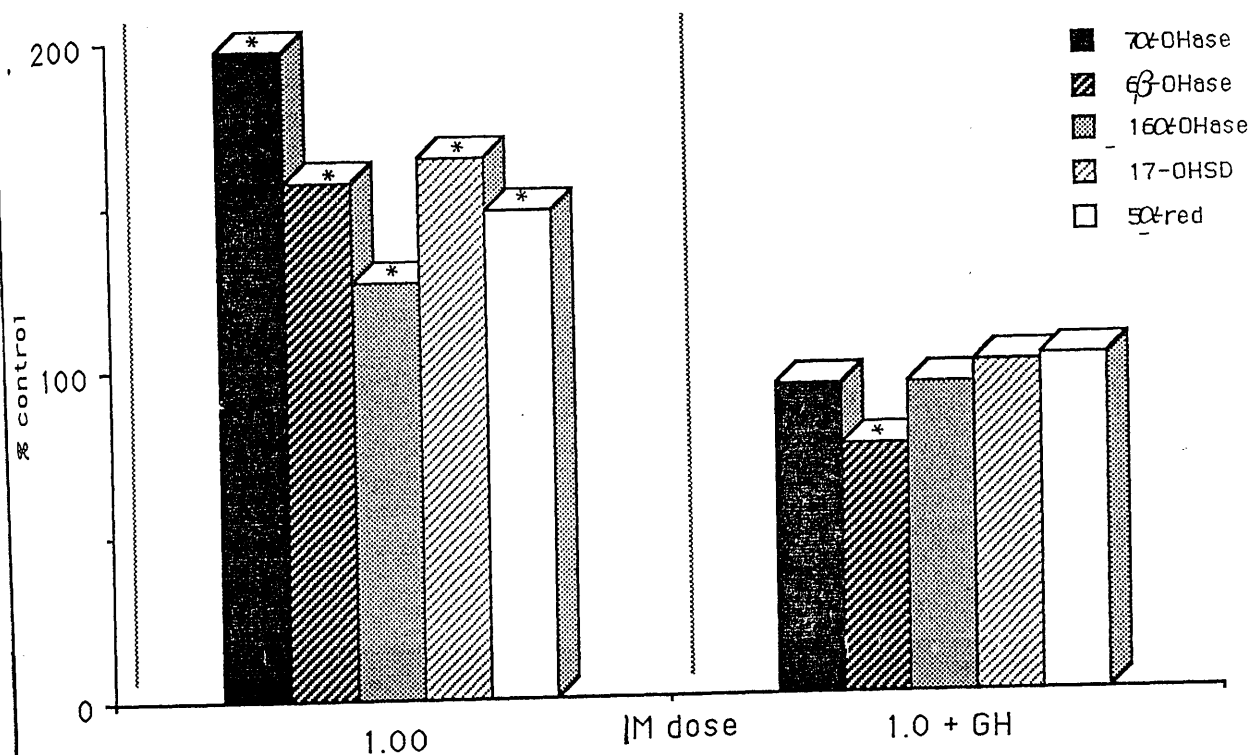


TABLE 38 : Effects of 30 minute preincubation of rat liver microsomes with 1:1 AG1x8-purified INSULIN MEDIATOR extract in the presence and absence of 10^{-9} M GROWTH HORMONE on 7 α -, 6 β -, 16 α -hydroxylases (OHases), 17-oxosteroid oxidoreductase (17-OHSD) and 5 α -reductase activities.

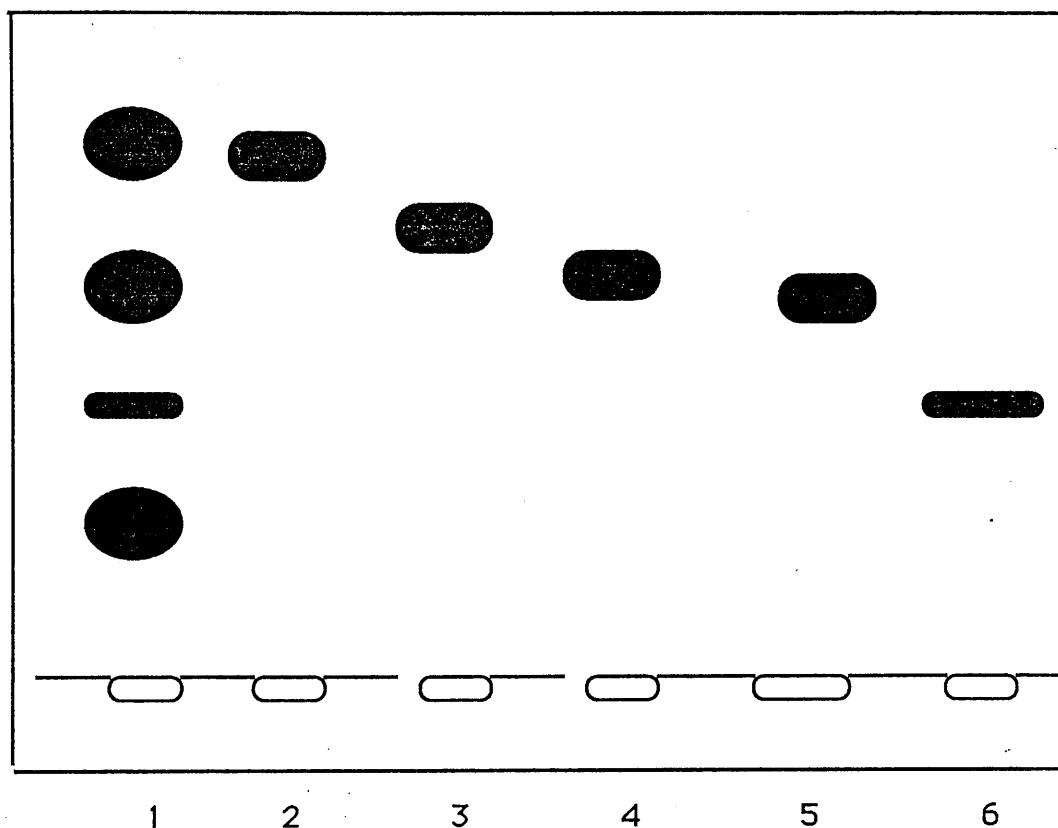
<u>TREATMENT</u>	<u>Enzyme activities (pmoles/minute/mg protein)</u>				
	<u>7α-OHase</u>	<u>6β-OHase</u>	<u>16α-OHase</u>	<u>17-OHSD</u>	<u>5α-red</u>
Control	39 \pm 3	77 \pm 7	93 \pm 4	99 \pm 7	101 \pm 9
IM	66 \pm 3*	88 \pm 4*	114 \pm 6*	136 \pm 14*	162 \pm 15*
Control	36 \pm 3	67 \pm 3	89 \pm 4	103 \pm 8	109 \pm 9
IM+GH	34 \pm 4	51 \pm 2*	84 \pm 2	103 \pm 5	111 \pm 7

Results are expressed as MEAN \pm S.D. (N=3); *=P<0.05 as compared to relevant controls.

which are thought to be the constituents of the insulin mediator (Larner 1987) were also applied in order to attempt to identify the components of the hydrolysed insulin mediator sample.

As is shown in Figure 60, several components were released upon hydrolysis of the insulin mediator extract. Although these components could be correlated with the standards used, exact identification was difficult since this technique did not distinguish significantly between the standard sugars applied. It can therefore be said that although carbohydrate components such as galactose, mannose, galactosamine, glucosamine and inositol do appear to be present in this insulin mediator extract, the exact nature requires a more sensitive technique.

FIGURE 60:CHROMATOGRAPHY OF THE AG1x8-PURIFIED,
ACID-HYDROLYSED INSULIN MEDIATOR EXTRACT.



- 1 :Insulin mediator extract
- 2 mannose
- 3 galactose
- 4 glucosamine
- 5 galactosamine
- 6 inositol

DISCUSSION

DISCUSSION

4.1 EXPERIMENTAL MODEL FOR THE STUDY OF HEPATIC STEROID METABOLISM

Many of the studies carried out on hepatic drug and steroid metabolism have employed in vivo animal models. Further, the endocrine influence on this system, studied either by administration of exogenous hormones, or by removal of a particular gland(s) has also been extensively documented. An obvious conclusion from these in vivo studies has been that no single hormone is involved in the regulation of this system, and hormonal interactions are known to exist (reviewed by Skett 1987). For instance, androgens and oestrogens effect their regulatory influence on hepatic metabolism by modulating the secretory pattern of GH secretion (section 1.3.2). It therefore became apparent at this stage that in vivo studies were not sufficiently adequate to evaluate the role of single hormones in this system.

In vitro microsomal preparations or reconstituted enzyme systems were subsequently used (Berg and Gustafsson 1973, Gustafsson and Stenberg 1974). Although the effects of single hormones can be determined using liver microsomes, whether or not the results are applicable to the physiological situation is questionable.

In the present study, we have used intact isolated hepatocytes. The advantages of this preparation are twofold. Firstly, the hepatocyte preparation is a relatively

physiological approach as compared to the situation with microsomal preparations in that the hepatocyte itself is not disrupted so that the components and enzymes of the cell are not interfered with (although cell-cell contact is obliterated). Secondly, the effects of single hormones on hepatic drug and steroid metabolism can be studied without the ambiguity of the hormonal interactions that may be encountered in vivo. In addition, precise combinations of hormones may be administered to attempt to mimic the situation in vivo under clearly defined culture conditions, thereby indicating which interactions may be taking place in the intact animal model.

Many studies have shown that isolated hepatocytes in culture do not maintain monooxygenase activity to levels found in vivo. However, numerous studies have subsequently reported that cytochrome P-450 content can be maintained in culture provided that the correct culture medium environment exists (Kremers et al 1981, Sinclair et al 1979, Doostdar et al 1988). Thus whereas cytochrome P-450 content and monooxygenase activity decreased during the first 48h in primary culture, activities were maintained at higher levels in hormone-supplemented media (Dickins and Paterson 1980). Cytochrome P-450 content was reported to be maintained at 65% of control levels for up to 2-3 weeks in culture in the presence of high doses of dexamethasone (Dich et al 1988). Similarly, cytochrome P-450 content in cultured female rat hepatocytes were maintained by low doses and induced by high doses of dexamethasone in the culture medium, although no effects were seen using male rat hepatocytes (Vind et al

1988). Further, the normal development of NADPH cytochrome P-450 reductase activity in cultured foetal hepatocytes was reported to be dependent on the presence of thyroid hormones in the culture medium (Kriz et al 1982).

Two additional culture systems were also shown to prevent the loss of cytochrome P-450 content in culture: Inclusion of a substituted pyridine such as nicotinamide (Villa et al 1988) and metyrapone or δ -aminolevulinic acid together with the exclusion of cystine and cysteine from the culture medium (Paine et al 1982, Lake and Paine 1982).

More recently, the long term culture of cytochrome P-450 activities in culture has been shown to be maintained by co-culturing hepatocytes with an epithelial cell line (Begue et al 1984) or on a basement membrane (Schuetz et al 1988). Dimethylsulphoxide was also reported to maintain certain hepatocyte functions such as albumin secretion (Isom et al 1985).

Hussin and Skett (1986) recently characterised a method for maintaining hepatocytes in a hormone-free culture system comprising Ham's F-10 culture medium supplemented with 0.1% bovine serum albumin and reported that cytochrome P-450 content was maintained at levels close to those expected in freshly isolated hepatocytes after removal of hormonal influences. We have therefore employed this technique in the present study, and although certain limitations do exist, it appears to be an essential method to study the effects of single hormones and the hormonal interactions involved in the regulation of hepatic steroid metabolism.

Hepatocytes from untreated male rats have been used

throughout this study. Firstly, since male rat hepatocytes contain exclusively somatogenic receptors (Ranke et al 1976), this is important in the dissociation of the somatogenic and lactogenic properties of hGH (Barash et al 1988). Secondly, the effects of diabetes, adrenalectomy and thyroidectomy appear only to be seen on male-specific enzymes and in male animals. Thus the effects of glucocorticoids, thyroxine and insulin should logically be studied in the male.

4.2 CHOICE OF SUBSTRATE

Androst-4-ene-3,17-dione was used as the substrate to monitor the effects of different hormones on the hepatic steroid-metabolising system. A number of advantages of using this substrate have been noted. Firstly, androstenedione is readily available in both a radio-labelled and unlabelled form. Secondly, androstenedione is metabolised by a number of enzymes (Sheets and Estabrook 1985) (Table 3). The 7 α -, 6 β -, and 16 α -hydroxylases are all cytochrome P-450-dependent enzymes, whereas the 17-oxosteroid oxidoreductase and 5 α -reductase are flavin dependent. Since androstenedione is metabolised by all five enzymes, which are readily detectable in male rat hepatocytes, the hormonal regulation of both cytochrome P-450-dependent and independent steroid metabolism can be studied. In addition, hepatic steroid metabolism is sexually differentiated in rats due to the presence of sex-specific enzymes (Waxman 1988). The 7 α -hydroxylase and 5 α -reductase are female-specific whilst

the 6 β - and 16 α -hydroxylase s and 17-oxosteroid oxidoreductase are male-specific (Table 1). Thus the feminisation (ie decrease in 6 β - and 16 α -hydroxylases and increase in 5 α -reductase activities) or masculinisation of steroid metabolism by treatment with hormones can be recognised depending on the metabolite profile of androstenedione. In addition, the metabolites of androstenedione have been previously characterised by gas chromatography-mass spectrometry and thin layer chromatography (Gustafsson and Stenberg 1974). Further, the regulation of androstenedione-metabolising enzymes has been widely documented by other groups (Waxman et al 1985, Waxman et al 1987, Waxman et al 1989a, Mode et al 1989).

4.3 EFFECTS OF INCUBATION OF HEPATOCYTES WITH GROWTH HORMONE

The regulatory role of GH in the maintenance of the sex differences observed in hepatic steroid metabolism in rats have been extensively documented (Waxman 1984). The secretory pattern of GH (Edén 1979) is now known to impose the sex differences observed in steroid metabolism such that the pulsatile male GH secretory pattern results in higher oxidative metabolism and the continuous pattern in females in higher reductase activity (Mode et al 1981). Thus continuous GH infusion to hypophysectomised rats resulted in the feminisation of steroid metabolism and intermittent GH administration led to an increased expression of male-specific activities together with a reduction in

female-specific enzymes (Mode et al 1982). These sex differences have also been determined at the biochemical level in that cytochrome P-450-male was increased upon intermittent GH administration and cytochrome P-450-female after continuous GH infusion (Kato et al 1986).

The results presented in this study, however, indicate that incubation of isolated hepatocytes with GH, that is, continuous GH exposure, exhibited no marked feminisation effects even at high GH concentrations (Figures 6 & 9). Time course studies with 10^{-9} M GH (Figure 7) showed a reduction in male-specific activities at the earlier time periods, but no increase in female-specific activities, which would be indicative of a feminisation effect, was observed. Exposure to GH over 24, 48 and 72h also exhibited no significant effects (Figure 8), except for slight decreases at 72h which may be attributable to inadequate culture conditions rather than an effect of GH.

In contrast to these findings, several groups have previously reported that continuous GH infusion in vivo resulted in the feminisation of microsomal steroid metabolism (Mode et al 1981, Kato et al 1986).

More recently, cytochrome P-450₁ levels (gene IIC12) were reported to increased upon treatment of male rat hepatocytes with GH, together with an increase in P-450₁ mRNA formation (Guzelian et al 1988). However, the corresponding 15 β -hydroxylase activity was not measured by these authors. The failure to "masculinise" steroid metabolism upon intermittent GH administration to female rats may reflect the requirement for other hormones in this

system. Thus it may be possible that the lack of effects of GH in isolated hepatocytes is as a direct result of the removal of other hormonal influences that may have been present in the intact animal.

The effects of growth hormone on hepatic drug metabolism were also reported to follow changes in NADPH cytochrome P-450 reductase activity rather than in cytochrome P-450 content (Wilson 1973).

Incubation of hepatocytes with GH exhibited no significant alterations in intracellular cyclic AMP content (Figures 10 & 11). Thus any effects of GH on this system are not mediated by the cAMP second messenger system. In contrast, previous attempts to elucidate the effects of GH on cAMP content in numerous systems have shown alterations in cAMP levels. Whereas GH appeared to act by reducing cyclic AMP levels in some reports (Thompson et al 1973, Payne and Kostyo 1970, Rillema et al 1973), elevations in intracellular cAMP were thought to mediate the effects of GH in other cases (Fain et al 1971, Sengupta et al 1981, Solomon et al 1987). In addition, other components of this system such as adenylate cyclase (Albertsson-Wikland and Rosberg 1982, Hughes and Friesen 1985) and cAMP phosphodiesterase (Payne and Kostyo 1970, Rillema et al 1973) were also reported to be altered by GH. More recently, the insulin-like activity of GH to stimulate glucose output in hepatocytes isolated from hypophysectomised rats was reported to be inconsistent with previous reports where a glucagon-like (adenylate cyclase) or insulin-like (tyrosine

kinase) mechanism was proposed for the GH action (Blake and Clarke 1988).

The lack of effects of GH on cAMP levels in this study could be due to a number of factors. Firstly, since GH itself exhibited no feminising effects under the present culture conditions, it may be that GH is inactive here, perhaps due to a lack of GH receptors, and thus no effects on cAMP content are observed. Van Neste et al (1988), however showed that GH receptors were present exclusively in liver parenchymal cell fractions. Ideally, intracellular cAMP content should be determined under conditions where GH exhibits an effect. If changes in cAMP content are involved in the reported actions of GH on steroid metabolism, the absence of these feminising effects of GH in this study correlates well with no changes in intracellular cAMP levels. In addition, elevations in intracellular cAMP were reported to non-specifically inhibit steroid metabolism in isolated hepatocytes, indicating that cAMP may not be involved in mediating the feminising effects of GH in this system (Weiner et al 1972, Berry and Skett 1988). Ros et al (1973) have, however, reported a sex-dependent inhibitory influence of cAMP on drug metabolism and cytochrome P-450 content. In agreement with the present study, cAMP levels were unchanged upon hypophysectomy or replacement GH therapy of male animals, indicating that the cAMP system may not be important in GH action (Isaksson et al 1974, Klingensmith et al 1980).

GH is known to stimulate the production of the low molecular weight somatomedin insulin-like growth factor-I

(IGF-I) from hepatocytes in culture (Scott et al 1985) and IGF-I mRNA levels (Norstedt and Möller 1987). In addition, hypophysectomy resulted in a reduction in IGF-I levels and in the expression of IGF-I mRNA (Scott et al 1985), that was restored upon continuous GH infusion (Isgaard et al 1988, Jansson et al 1982, Clark et al 1985).

If IGF-I is responsible for mediating the effects of GH on steroid metabolism, the lack of feminising effects of GH may be due to an inability of GH to stimulate IGF-I production from the liver cells. Cultured hepatocytes have been shown to be responsive to IGF-I production stimulated by GH (Spencer 1979). Direct administration of IGF-I to isolated hepatocytes, however, resulted in a general stimulation of steroid metabolism in a dose-dependent manner (Figure 12), which is characteristic of an insulin-like effect (Hussin and Skett 1987) rather than the sex-specific effect expected of GH. In agreement with this result, the somatomedins are reported to mimic many of the effects of insulin (Froesch et al 1985). Thus the effects of GH on steroid metabolism do not appear to be mediated by IGF-I. In accordance with this finding, Guzelian et al (1988) have recently reported that the induction of cytochrome P-450₁ content (gene IIC12) by GH was not mediated by IGF-I since IGF-I administration did not mimic the effects of GH on P-450₁ expression.

In conclusion, the feminising effects of GH observed in vivo were not exhibited upon incubation of isolated hepatocytes with GH alone. The absence of any effects of GH under these conditions on intracellular cAMP shows that the

CAMP system may not be the second messenger system involved in GH action on steroid metabolism. However, this result may be as a direct consequence of the apparent inactivity of GH in isolated hepatocytes. IGF-I does not appear to be involved in mediating the effects of GH on steroid metabolism, but rather exhibits an insulin-like effect.

4.4 EFFECTS OF INSULIN ON STEROID METABOLISM AND ITS INTERACTION WITH GROWTH HORMONE

A number of reports have indirectly demonstrated that insulin plays an important role in the regulation of drug metabolism since diabetic rats exhibited marked changes in drug metabolism by hepatic enzymes (Kato and Gillette 1965). In addition, the diabetic state was reported to cause a feminisation of steroid metabolism, which implied that insulin may be acting as a "masculinising" factor (Skett 1986). Incubation of hepatocytes with insulin resulted in the marked stimulation of all enzyme activities in a sex-independent manner (Hussin and Skett 1987), although the diabetic state appears only to affect male-specific activities (Kato and Gillette 1965, Skett and Joels 1985).

In the present study, insulin stimulated steroid metabolism in a dose-dependent manner with maxima at physiological concentrations (Figure 13), as has been shown previously by Hussin and Skett (1987). Incubation of hepatocytes with insulin in combination with GH (Figures 13-16) showed that GH antagonised the stimulatory effects of insulin in a non-specific manner. Further, no effects of GH

were observed in the absence of an insulin response, indicating that GH was indeed opposing insulin action. Thus, although GH alone exhibited no significant effects on steroid-metabolising enzymes, GH modified the effects of insulin, indicating that the hepatocyte culture system was functional and could respond to GH.

Insulin and GH are known to interact in numerous systems and, insulin, like GH, is also known to stimulate IGF-I release from the liver (Maes et al 1983). Conversely, GH also exhibits insulin-like effects in many systems (Betley et al 1989). Serum IGF-1 levels were low in insulin-dependent diabetics and were not restored upon GH administration (Maes et al 1986). This GH-resistance was thought to be due to a post-receptor defect since GH-binding was not altered in diabetic animals.

The GH secretory pattern was markedly suppressed in the diabetic animal, and led to the abolition of the pulsatile GH secretion in normal males (Harrison and Robinson 1980, Tannenbaum 1981). Thus removal of the insulin influence resulted in the feminisation of the GH secretory profile, in agreement with the reported feminisation of steroid metabolism (Skett 1986). Insulin administration should therefore have resulted in a "masculinisation" of the steroid metabolising enzymes. However insulin stimulated steroid metabolism in a non-specific manner (Figure 13), showing that additional factors may also be involved. Co-incubation with GH modified the effects of insulin in a non-sex-specific manner since both male- and female-specific activities were decreased. Thus although the effects of

insulin are antagonised by GH, other interactions appear to be involved in achieving the feminisation of steroid metabolism typical of continuous GH administration to the intact animal (Mode et al 1981).

Both native and recombinant bGH, but not bPRL antagonised the insulin-stimulated lipogenesis in cultured adipose tissue, showing that this effect was specific to GH (Etherton et al 1987). In addition, the effects of GH were augmented in the presence of hydrocortisone, showing the involvement of other hormones. It has further been reported that streptozotocin (STZ)-diabetes enhanced the sensitivity to lipolytic agents such as GH (Solomon et al 1987). Conversely, insulin sensitivity was enhanced in rats treated with a GH antibody (Schwartz 1980, Gause et al 1983). Tatro and Schwartz (1987) showed that endogenous GH suppressed glucose utilisation in diabetic animals, which was reversed by prolonged treatment with a GH antibody. Similarly, the interaction between insulin and GH was documented by the observed increase in sensitivity to insulin in hypophysectomised rats (Schwartz 1980).

The stimulatory effects of insulin on steroid metabolism did not appear to be as a result of de novo cytochrome P-450 synthesis since total cytochrome P-450 content remained unchanged in insulin-treated hepatocytes (Hussin and Skett 1987). However alterations in the levels of specific cytochrome P-450 isoenzymes cannot be excluded as a possible mechanism. The mechanism of the stimulatory action of insulin on steroid-metabolising enzymes was postulated to involve a phosphorylation reaction since

incubation with insulin of hepatocytes pretreated with protein kinase inhibitors resulted in the abolition of the insulin response (A.H. Hussin, personal communication).

The cytochrome P-450 enzymes have been shown to be substrates for phosphorylation. The catalytic subunit of cyclic AMP-dependent protein kinase (Pyerin et al 1983), both in a reconstituted system and in purified form, phosphorylated cytochrome P-450 LM₂ (gene IIB4) and cytochrome P-450 reductase from rabbit liver microsomes, resulting in an associated decrease in monooxygenase activity and conversion of cytochrome P-450 to inactive cytochrome P-420 (Pyerin et al 1984). It was further shown that phosphorylation of cytochrome P-450 occurred exclusively on serine residues, namely ser 128 (Pyerin et al 1986). Subsequent studies have categorised the phosphorylation of cytochrome P-450 into three groups : cytochrome P-450 isoenzymes that are phosphorylated by both cAMP-dependent protein kinase (protein kinase A) and calmodulin/phospholipid-dependent protein kinase (protein kinase C), cytochrome P-450 isoenzymes that are phosphorylated by either protein kinase A or protein kinase C, and isoenzymes not phosphorylated by either protein kinase (Pyerin et al 1987).

In agreement with the studies carried out by Pyerin and coworkers, Koch and Waxman (1989) have recently reported the phosphorylation of cytochromes P-450 PB-4 (gene IIB1) and PB-5 (gene IIB2) by dibutyryl-cAMP (and therefore by cyclic AMP-dependent protein kinase activation) at ser 128. The failure to achieve the phosphorylation of other cytochrome

P-450 isoenzymes was attributed to either the involvement of protein kinase C or the absence of a serine residue at position 128, which is contained within a recognition sequence (Arg-Arg-x-Ser) used by many cyclic AMP-dependent protein kinases (Krebs and Beavo 1979).

It has been suggested that the phosphorylation sites of cytochrome P-450 may be closely associated with the cytochrome b₅ binding domain since the effects of protein kinases A and C activation on cytochrome P-450 phosphorylation were inhibited by cytochrome b₅ (Epstein et al 1989).

Phosphorylation-dephosphorylation control by insulin has been well documented in numerous systems. Thus altered phosphorylation of cytochrome P-450 caused by insulin could result in a stimulation of enzyme activity. Conversely, the actions of glucagon to inhibit steroid metabolism correlated well with a diacylglycerol-mediated activation of protein kinase C, which then presumably phosphorylates cytochrome P-450 (Hussin et al 1988). Incubation of rat liver microsomes with alkaline phosphatase, however, also resulted in a decrease in monooxygenase activity (Taniguchi and Pyerin 1987), which is similar to the effects of cytochrome P-450 phosphorylation (Pyerin et al 1987). This discrepancy was resolved by the finding that alkaline phosphatase was acting to inactivate NADPH cytochrome P-450 reductase, rather than at the level of cytochrome P-450 (Taniguchi and Pyerin 1987).

The effects of GH to antagonise the stimulatory effects of insulin could also involve control at the phosphorylation

level. Certainly, GH was shown to cause the phosphorylate a 46kDa species in isolated rat hepatocytes (Yamada et al 1987). Thus GH could be acting to phosphorylate cytochrome P-450 isoenzymes, which would result in a decrease in monooxygenase activity. Which protein kinase could be activated by GH to mediate cytochrome P-450 phosphorylation (Pyerin et al 1987) is not clear, since GH has been shown to activate both cAMP and PKC systems (Thompson et al 1973, Albertsson-Wikland and Rosberg 1982, Smal et al 1989, Doglio et al 1989), although no effects on cyclic AMP were observed in this study (Figures 10 and 11).

Insulin is also known to phosphorylate at serine and threonine residues (Espinal 1987). Activation of cytochrome P-450 activity by insulin could be mediated by activation of specific serine phosphatases (Czech 1988), and antagonism of this effect of insulin by GH could be due to phosphorylation at the level of cytochrome P-450, as a consequence of activation of PKA and/or PKC by GH. In accordance with this, it has been shown previously that increases in intracellular cAMP or PKC activation are associated with decreases in hepatic steroid (Berry and Skett 1988, Allan and Skett 1988) and drug (Weiner et al 1973, Banheygi et al 1988) metabolism.

Hypophysectomy has been reported to result in the increased expression of cytochrome P-450_j (gene IIE1), an effect that was reversed upon continuous GH administration (Williams and Simonet 1988). Thus GH appeared to act as a repressive factor for constitutive cytochrome P-450_j levels (Waxman et al 1989b). Cytochrome P-450_j (gene IIE1)

induction has also been reported in diabetic animals (Past and Cook 1982), and so the observed interaction between GH and insulin in this study may be as a result of modulations of cytochrome P-450_j content. The mechanism of this repressive action of GH is, as yet, not fully understood.

In conclusion, insulin appears to be a general stimulatory factor of steroid metabolism, an effect that is strongly antagonised by GH. This interaction between insulin and GH was optimal at physiological concentrations of insulin, and GH modulated enzyme activity in a sex-independent manner. Although the mechanism of this interaction is not fully clear at this stage, direct effects at the level of cytochrome P-450 are a strong possibility.

4.5 EFFECTS OF DEXAMETHASONE ON STEROID METABOLISM AND ITS INTERACTION WITH GROWTH HORMONE

The permissive role of glucocorticoids has been demonstrated in numerous systems. Further, adrenalectomy resulted in a reduction in male-specific enzyme activities (Gustafsson and Stenberg 1974d, Kato and Gillette 1965). Administration of glucocorticoids to normal animals has given rise to a number of conflicting reports. Whereas the long acting synthetic glucocorticoids markedly stimulated enzyme activity, corticosterone administration resulted in the opposite effect (Tredger et al 1976). As these studies were carried out in vivo, the final effect could have been as a result of interactions with other factors.

In the present study, a low concentration of

dexamethasone was added to isolated hepatocytes over varying time periods and in both the absence or presence of GH. Dexamethasone alone stimulated steroid metabolism non-specifically (Figures 17 and 18) over short incubation periods, while inclusion of GH in the culture medium restored enzyme activity to control levels (Figure 17). In addition, a certain degree of dose-dependency of GH was exhibited in modulating the response to dexamethasone (Figure 18). Incubation of cultured hepatocytes with dexamethasone over longer time periods resulted in a marked stimulation of all enzyme activities with time (Figure 19), and were decreased to control levels upon further incubation with GH (Figures 20-25).

Thus the results obtained from the present study show that dexamethasone stimulates steroid metabolism non-specifically over both short and long incubation periods, and that the GH response is modified in the presence of dexamethasone. The mechanism of action of these hormones remains obscure at this stage. While the short incubation studies probably represent a glucocorticoid function of dexamethasone, the mechanism of action in the longer term cultures may represent induction. The presence of dexamethasone in the culture medium has previously been shown to be important in the maintenance of the components of the cytochrome P-450 system in vitro to levels comparable with those in the intact animal (Kriz et al 1982, van der Hoeven and Guzelian 1987, Dich et al 1988, Vind et al 1988). However the culture medium used in the present study was shown to be adequate for maintaining cytochrome P-450

content in isolated hepatocytes (Hussin and Skett 1986), and so the observed effects of dexamethasone in cultured hepatocytes are thought to represent induction rather than maintenance alone.

Schuetz et al (1984) have also reported a similar response upon incubation with dexamethasone and was dependent on the age of the culture. Whereas dexamethasone added to fresh cultures (0 days) led to a 70% increase by 120h, dexamethasone added to 48h cultures resulted in a 20-25% induction of cytochrome P-450_{PCN} (gene IIIA1) by 120h (Schuetz et al 1984). In the present study, hepatocytes were exposed to dexamethasone after 48h of incubation (see section 2.5.2). The response between the two studies shows a striking similarity and so the results obtained in this study may therefore also represent cytochrome P-450_{PCN} induction.

Thus low concentrations of dexamethasone induced steroid-metabolising enzymes in this study, and it has been shown that this induction is a dose-dependent effect (Schuetz et al 1984). In addition, natural glucocorticoids also induced cytochrome P-450_{PCN} (gene IIIA1), although this effect was not as potent as with dexamethasone and required higher concentrations in order to achieve a reasonable response (Schuetz et al 1984). This effect may be as a consequence of the shorter half-life of the natural glucocorticoids in comparison to the synthetic analogues, as has been suggested previously by Tredger et al (1976).

In contrast, Mathis et al (1986) have reported that although dexamethasone induced monooxygenase activity

synergistically with polycyclic aromatic hydrocarbons upon incubation of cultured human foetal hepatocytes for 5 days, whereas dexamethasone alone exhibited no stimulatory effects. Further, these authors showed that dexamethasone was acting by the classical glucocorticoid receptor pathway and not by cytochrome P-450_{PCN} (gene IIIA1) induction.

Hepatocytes incubated in the presence of pregnenolone-16 α -carbonitrile (PCN) resulted in marked increases in cytochrome P-450_{PCN} (gene IIIA1) content, drug metabolism and mRNA levels (Elshourbagy et al 1981). It was subsequently shown that dexamethasone and other glucocorticoids also stimulated de novo synthesis of cytochrome P-450_{PCN} (gene IIIA1) (Althaus and Meyer 1981). Further, dexamethasone appeared to be the most potent inducer of cytochrome P-450_{PCN} (gene IIIA1) (Schuetz et al 1984). It was therefore proposed that both endogenous and synthetic glucocorticoids and PCN, but not sex steroids or mineralocorticoids, constituted the third class of inducers (Schuetz et al 1984) responsible for the induction of the steroid-inducible cytochrome P-450s of the P-450III gene family (Gonzalez 1989). Multiple isoenzymes of steroid-inducible cytochrome P-450_{PCN} (gene IIIA1) have subsequently been isolated (Graves et al 1987, Halpert 1988). Dexamethasone also induced specific cytochrome P-450 isoenzymes mediating the metabolism of 2-acetylaminofluorene in cultured rat hepatocytes (McManus et al 1987). A glucocorticoid-inducible cytochrome P-450 in human foetal livers that is related to cytochrome P-450 HLP (gene IIIA3) in adult human livers has also been identified (Wrighton et

al 1988).

The mechanism of action of glucocorticoids in the induction of cytochrome P-450_{PCN} (gene IIIA1) was subsequently reported to involve a mechanism distinct from the classical glucocorticoid receptor pathway. Firstly, tyrosine aminotransferase activity, a glucocorticoid-responsive function, showed no similarities in its induction as compared to cytochrome P-450_{PCN} (gene IIIA1) induction with regards to either the time-course of induction or the concentration of glucocorticoid required for this induction (Schuetz and Guzelian 1984). The most convincing piece of evidence to support these results has been that whereas tyrosine aminotransferase activation was abolished in the presence of glucocorticoid antagonists, no effect was observed on the magnitude of cytochrome P-450_{PCN} (gene IIIA1) induction (Schuetz and Guzelian 1984). Thus although de novo cytochrome P-450_{PCN} (gene IIIA1) synthesis appears to be involved in response to both glucocorticoids and PCN (Schuetz et al 1984), the mechanism of action appears to be other than that of the classical glucocorticoid receptor pathway (section 1.6) However, a glucocorticoid-responsive element (GRE) which binds the glucocorticoid receptor has been identified within the first intron of the cytochrome P-450_c gene (IA1), thus implying a direct regulation of the rat cytochrome P-450_c gene expression by glucocorticoids (Mathis et al 1989). Deletion of these glucocorticoid receptor binding sites within intron I led to the abolition of glucocorticoid responsiveness. These authors proposed that glucocorticoids may therefore

play an important role in the regulation of cytochrome P-450_c (gene IA1) to other xenobiotics and inducers. A direct action of glucocorticoids on the liver has also been implicated by the finding that cortisone acetate administration to hypophysectomised rats resulted in a decrease in cytochrome P-450_b (gene IIB1) and P-450_e (gene IIB2) content (Yamazoe et al 1989).

The administration of adrenocorticotropin (ACTH), which stimulates the release of glucocorticoids from the adrenal glands, in combination with GH to hypophysectomised animals resulted in a decrease in the reductive metabolism of corticosterone whereas either of the hormones alone exhibited no such effects (Colby et al 1974). This result implied that hormonal interactions may be important in hepatic drug and steroid metabolism.

Cytochrome P-450_b (gene IIB1) induction by dexamethasone has been suggested to be due to the stabilisation of its mRNA (Whitlock 1986). Rats treated with dexamethasone showed that mRNA levels for IIB1, IIB2 and NADPH cytochrome P-450 reductase were specifically stabilised in the absence of transcription (Simmons et al 1987). In contrast, Watkins et al (1986) reported that the PCN-1 gene was induced at the transcriptional level and that mRNA levels correlated well with PCN-1 protein content. The rat PCN-1 and PCN-2 proteins were also stabilised by the macrolide antibiotic triacetyloleandomycin (TAO), although by a mechanism distinct from that of dexamethasone (Watkins et al 1986, Halpert 1988).

In the present study, GH restored the dexamethasone-

induced stimulation of enzyme activity to control levels. GH is known to act as a regulatory factor in the development of a number of cytochrome P-450 isoenzymes (section 1.4.3). Thus expression of cytochrome P-450_{16 α} (gene IIC11) at puberty is as a direct consequence of the pulsatile secretory pattern of GH in males, while cytochrome P-450_{15 β} (gene IIC12) and 5 α -reductase activities are expressed in adult females following the development of a continuous GH secretory pattern. Conversely, the expression of cytochrome P-450_{6 β} (gene IIIA2) is suppressed by continuous GH secretion in females at the onset of puberty, and remains at prepubertal levels in adult males. It has further been suggested that maintenance of cytochrome P-450_{6 β} expression in adult males may also be partially dependent on the presence of adrenal corticosteroids (Waxman 1988), which correlates with the relatively specific effects of adrenalectomy on male-specific activities (Gustafsson and Stenberg 1974d).

More recently, an additional regulatory role of GH other than that exerted by its pulsatile secretory pattern has become apparent. Both cytochromes P-450 RLM2 (gene IIB4) and 2a (gene IIIA2) were suppressed upon GH administration to male rats, which would explain their relative absence in adult females (Waxman et al 1988a). Both constitutive and inducible levels of cytochromes P-450b (gene IIB1) and P-450e (gene IIB2) are regulated repressively by GH (Yamazoe et al 1987), as well as cytochrome P-450_{6 β} (gene IIIA2) expression (Yamazoe et al 1986). In addition, it was reported that the repressive effect of GH on these two

isoenzymes was distinct from the regulatory influence exerted by its pulsatile secretion in that hypophysectomy abolished the sex differences by increasing both cytochrome P-450b and P-450e expression in phenobarbital-treated female rats (Yamazoe et al 1987). Similarly, no sex-related difference was observed in animals given large doses of dexamethasone, which is known to reduce serum GH in rats (Kokka et al 1972).

It has been suggested that GH may regulate cytochrome P-450b (gene IIB1) and P-450e (gene IIB2) synthesis both at the transcriptional and translational level, as has been suggested for $\alpha_2\mu$ -macroglobulin (Kulkarni et al 1985). Cytochrome P-450b (gene IIB1) and P-450e (gene IIB2) were present at higher levels in 20-day old rats as compared to mature rats, which is consistent with P-450b mRNA levels (Giachelli et al 1986), and correlates well with the lower serum GH levels in 20-day old rats (Wehrenberg 1986). Cytochrome P-450b (gene IIB1) was reported to be more responsive to GH than cytochrome P-450e (gene IIB2) (Yamazoe et al 1987), which may partly be explained by the regulation of these isoenzymes by different genetic loci (Rampersaud and Walz 1983). Dexamethasone was reported to inhibit the GH-induced stimulation of IGF-1 mRNA in hypophysectomised rats and controls (Luo and Murphy 1989). Hence inhibition of IGF-1 mRNA gene expression may be the mechanism by which glucocorticoids inhibit growth, since IGF-1 is known to mediate the effects of GH on somatic growth (Jansson et al 1985). This mechanism is not likely with steroid metabolism, however, as GH does not appear to act via IGF-1 here

(section 4.3) (Guzelian et al 1988).

Thus the mechanism of action of the stimulatory action of dexamethasone could be either by the classical glucocorticoid receptor pathway (Mathis et al 1989) or by induction of cytochrome P-450_{PCN} (gene IIIA1) (Schuetz et al 1984). The interaction with GH could be due to the repressive effect of GH that has been reported on the expression of a number of cytochrome P-450 isoenzymes (Yamazoe et al 1987, Waxman et al 1988).

In conclusion, dexamethasone acts as a non-specific stimulatory agent of steroid metabolism, an effect that is restored to basal levels upon incubation with GH. The mechanism of action of this suppressive effect is not clear, although GH has been documented to be a repressive factor for many cytochrome P-450 isoenzymes. Thus it is unlikely that a combination of GH and glucocorticoid can account for the feminising effect of GH seen in vivo.

4.6 EFFECTS THYROXINE ON STEROID METABOLISM AND ITS INTERACTION WITH GROWTH HORMONE

The role of the thyroid hormones in the regulation of hepatic drug and steroid metabolism has been noted indirectly by the effects of thyroidectomy. Kato and Takahashi (1968) showed that thyroidectomy resulted in a reduction in drug metabolism and in the activities of other components of the cytochrome P-450 system in both male and female rats. Administration of thyroid hormones exhibited sex-and substrate-dependent effects on drug metabolism (Kato

and Gillette 1965, Rumbaugh et al 1978, Skett and Weir 1983, Colby and Rumbaugh 1985), although a number of discrepancies have been documented regarding the administered dose of thyroid hormones.

In the present study, thyroxine exhibited a dose-related and sex-specific effect on steroid metabolism (Figure 26 and 27). Whereas short incubation with thyroxine alone exhibited isoenzyme-specific effects, incubation with GH resulted in marked decreases in enzyme activities (Figure 29). Incubation over longer time periods with thyroxine alone caused no significant effects, except at 72h with 10^{-6} M thyroxine, and enzyme activities were in general restored to control levels upon further incubation with GH (Figures 30-43). The nature of the discrepancy observed in GH action between short and long incubation periods indicates that GH may be acting by a different mechanism in each case.

Thyroid hormones, similar to steroid hormones (King 1987), interact with nuclear receptors and then exert their effects by binding to thyroid hormone responsive elements (TRE) on target genes (section 1.7).

The mechanism of action of the thyroid hormones was suggested to be dependent on the presence of androgens (Kato and Takahashi 1968). Thus supraphysiological doses of thyroxine antagonised the stimulatory effects of androgens on hepatic microsomal metabolism, but in the absence of androgens, thyroxine increased monooxygenase activity. Such a mechanism would account for the sex-specific effects of thyroxine observed by these authors. In contrast,

physiological doses of thyroxine stimulated drug metabolism in a sex-independent manner irrespective of pituitary (Rumbaugh and Colby 1978) or gonadal status (Colby and Rumbaugh 1985). However, Skett and Weir (1983) demonstrated sex- and substrate-dependent effects of physiological doses of thyroxine, and further suggested that an optimal concentration of thyroxine existed that was responsible for maximum activity, in which case the discrepancies amongst the above reports can be accounted for purely on the basis of the use of different concentrations of thyroid hormone. This is further supported by a recent report where physiological concentrations of thyroid hormone induced δ -aminolaevulinate synthase activity (therefore increased cytochrome P-450 synthesis) while thyrotoxic doses induced haem oxygenase activity (therefore increased cytochrome P-450 degradation) (Smith and Drummond 1988).

The mechanism by which thyroxine elicits these effects on drug metabolism may be as a direct result of alteration in other components of the cytochrome P-450 system. The thyroid hormones have recently been shown to be important regulatory agents for the constitutive levels of cytochrome P-450 isoenzymes in male rats (Arlotto and Parkinson 1989), since thyroidectomy elevated microsomal 7 α -hydroxylase activity which was restored to control levels upon replacement triiodothyronine therapy. In addition, cytochrome P-450b (gene IIB1) and P-450e (gene IIB2) content was markedly suppressed upon administration of triiodothyronine to hypophysectomised rats, indicating a regulatory role of the thyroid hormones in the control of

specific cytochrome P-450 forms (Yamazoe et al 1989). Liver microsomal cytochrome b_5 , NADPH cytochrome P-450 reductase and haem oxygenase have been shown to be partially regulated by triiodothyronine (Hoch et al 1980, Smith et al 1982, Phillips and Langdon 1962, Rumbaugh et al 1978). Further, the stimulatory effects of thyroxine on hepatic drug metabolism was reported to correlate with changes in NADPH cytochrome P-450 reductase activity rather than with cytochrome P-450 content (Colby and Rumbaugh 1985). A similar dependence on NADPH cytochrome P-450 reductase levels in cytochrome P-450-dependent metabolism has recently been documented (Waxman et al 1989a).

In contrast to the present studies, increases in 5 α -reductase activity by GH were not modified upon inclusion of triiodothyronine in the incubation medium. However, this study was carried out using female rat hepatocytes, so the effect with GH is not a feminisation response (Miller and Colás 1982). A permissive action of thyroid hormones on the actions of hormones that act via cyclic AMP has been documented by Malbon et al (1988). Many of the actions of GH have been associated with the cyclic AMP system and so the modulation of the GH response in the presence of thyroxine may be as a direct result of this permissive action. In accordance with this, a cAMP-responsive region in the rat GH gene was reported to be synergistically activated by thyroid hormones and cAMP (Copp and Samuels 1989).

Thyroxine may directly affect GH at the transcriptional level since TRE have been identified on the GH gene (Ye and Samuels 1987). Triiodothyronine stimulates the transcription

of the GH gene in the pituitary (Flug et al 1987). Thus T_3 could possibly act through the hypothalamo-pituitary axis by modulation of the GH-dependent process. Thyroxine has been reported to increase the synthesis of GH by the pituitary gland (Mirell et al 1987, Santos et al 1987). However, T_3 appears to act as a repressive factor directly in the liver and not via the pituitary in this case since triiodothyronine exhibited suppression in hypophysectomised rats (Yamazoe et al 1989). Regulation at the level of the pituitary is an unlikely explanation for the effects seen in this study, however, as isolated hepatocytes were used (and therefore no synthesis of GH is taking place).

In conclusion, short incubation of hepatocytes with thyroxine altered steroid metabolism in a dose-dependent and sex-related manner. Co-incubation with GH exhibited marked decreases in enzyme activity as compared with the results obtained with thyroxine alone. Longer term culture of hepatocytes with thyroxine and GH did not exhibit significant decreases in enzyme activity, except at 72h of incubation, and there was a tendency of GH to restore steroid metabolism to control levels. Thus, as with the previous section, it is unlikely that a combination of GH and thyroid hormone can account for the feminising effect of GH in vivo.

4.7 EFFECTS OF DEXAMETHASONE AND THYROXINE ON STEROID METABOLISM AND THEIR INTERACTION WITH GROWTH HORMONE

Thyroid hormones and glucocorticoids have been documented to be permissive factors in mediating the effects of numerous other systems. In the present study, the effects of incubation with thyroxine or dexamethasone were altered in the presence of GH, indicating a certain degree of interaction between these hormones (see above).

Although the continuous infusion of GH to hypophysectomised male rats resulted in the feminisation of hepatic steroid metabolism (Mode et al 1981), this effect was only achieved in the presence of both ACTH and thyroxine in another study (Rumbaugh and Colby 1980). It was subsequently suggested by these authors that the lack of effects of GH in isolated hepatocytes may have been due to inappropriate culture conditions, that is, the apparent requirement for ACTH and thyroxine was not fulfilled in these in vitro experiments.

Duran-Garcia et al (1979) reported that the number, but not affinity of GH binding sites in liver was markedly reduced in thyroidectomised rats, and restored upon replacement with thyroid hormone, suggesting that thyroid hormones may be required for the maintenance of GH receptors. Similarly, the role of dexamethasone and insulin has also been documented in the maintenance of GH binding sites in vitro (Christoffersen et al 1984). Thus it may be that the role of dexamethasone and thyroxine is not in cytochrome P-450 maintenance, but rather with the

maintenance of the GH receptor in isolated hepatocytes.

As was discussed in section 4.2, incubation of hepatocytes with GH gave no significant feminisation of steroid metabolism. In addition, hepatocytes incubated with either dexamethasone or thyroxine in combination with GH exhibited non-specific effects and feminisation of steroid metabolism was not achieved.

Incubation of hepatocytes with dexamethasone and thyroxine caused no significant effects on enzyme activities (Figures 44 and 46), which is consistent with the maintenance of cytochrome P-450 levels in hepatocytes cultured in hormone-supplemented media (Vind et al 1988, Dich et al 1988). In addition, hepatocyte NADPH cytochrome P-450 reductase activity was maintained at original levels in the presence of dexamethasone, triiodothyronine and insulin while either of the hormones alone exhibited no significant effects (van der Hoeven and Guzelian 1987).

Incubation with GH in the presence of dexamethasone and thyroxine in the culture medium resulted in an increase in female-specific and a decrease in male-specific enzyme activities (Figures 44 and 46), which is characteristic of the feminisation of steroid metabolism (Mode et al 1981). In addition, the higher dose of GH more potently decreased male-specific activities whereas 10^{-9} M GH was more effective in the stimulation of female-specific enzymes (Figure 45). This differential effect of the dose of GH on male- and female-specific activities probably reflects the sexually-differentiated secretory pattern of GH in vivo. Thus both dexamethasone and thyroxine appear to be important

in manifesting the feminising effects characteristic of GH in cultured hepatocytes. Similarly, Mode et al (1989) have recently reported that the feminisation of cytochrome P-450_{15 β} (gene IIC12) levels (female-specific) upon continuous GH administration to hypophysectomised rats was markedly potentiated in the presence of thyroxine and cortisol in a synergistic manner. In contrast, the masculinisation of cytochrome P-450_{16 α} (gene IIC11) by intermittent GH administration was unaffected by the presence of additional hormones. Thus thyroid hormones and glucocorticoids appear to be important in the maintenance of a "female-type" liver (Mode et al 1989). Contrary to this report, however, GH alone increased 5 α -reductase activity in cultured female rat hepatocytes, an effect that was not potentiated in the presence of dexamethasone and/or triiodothyronine (Miller and Colás 1982).

A recent study carried out in adult female rat hepatocytes cultured on matrigel showed that administration of GH resulted in the increased expression of cytochrome P-450₁ (gene IIC12) and its mRNA. IGF-I mRNA levels were also stimulated. In contrast, treatment of female rat hepatocytes with GH and other hormones did not induce cytochrome P-450_h (gene IIC11) expression (Guzelian et al 1988). Since continuous GH did not increase cytochrome P-450_{15 β} (gene IIC12) content to that found in control animals, together with the lack of masculinisation of cytochrome P-450_h (gene IIC11) in females, this indicated that other factors may also be involved (Guzelian et al 1988). These authors subsequently reported the need for

glucocorticoids and thyroid hormones in order to achieve the same effect in hypophysectomised rats (Mode et al 1989). An additional discrepancy in these studies has been that insulin is present both in hypophysectomised rats and especially in the matrigel culture medium used by Guzelian et al (1988). The effects observed may therefore not reflect the true response to GH alone. Further, cytochrome P-450_{15β} (gene IIC12) expression was not induced by GH to levels found in normal control animals, suggesting that additional factors may also be involved (Zaphiropoulos et al 1988).

The mechanism of action by which GH elicits the feminisation of hepatic steroid metabolism in the rat is not known. Pretranslational control has been suggested in the induction of cytochrome P-450_{15β} (gene IIC12) by GH (Mode et al 1989, Guzelian et al 1988) since associated mRNA levels were also increased in response to GH. GH was reported to have reduced the total amount of cytochrome P-450 in hypophysectomised male rats (Vockentanz and Virgo 1985). By labelling cytochrome P-450 with ³H-haem, it was shown that GH decreased the amount of cytochrome P-450-male (slow turnover) markedly as compared to cytochrome P-450-female (fast turnover) and increased the half-life of cytochrome P-450-female to a greater extent. The authors therefore proposed that the feminising effects of GH were as a result of a decrease in the synthesis of cytochrome P-450-male together with an increase in the half-life of cytochrome P-450-female (Vockentanz and Virgo 1985).

The feminisation of hepatic cytochrome P-450 expression by cisplatin was reported to be as a result of depletion of

serum androgens which are required for the maintenance of male-specific activities (LeBlanc and Waxman 1988).

Although a number of actions of GH have been associated with changes in intracellular cyclic AMP content (Thompson et al 1973, Payne and Kostyo 1970, Rillema et al 1973, Fain et al 1971, Sengupta et al 1981, Solomon et al 1987) no conclusive second messenger of GH has been identified as yet. GH did not alter cAMP levels in the present study, and IGF-I administration exhibited an insulin-mimetic effect (section 4.3). However, IGF-I is now generally believed to mediate the actions of GH on skeletal growth (Jansson et al 1985). Further, intermittent rather than continuous GH administration stimulated bone growth more effectively in hypophysectomised rats (Isgaard et al 1989). Recent studies on intracellular cyclic AMP led to the conclusion that agents elevating intracellular cyclic AMP (such as adrenaline, phosphodiesterase inhibitors, cyclic AMP analogues) resulted in marked inhibitions of steroid metabolism (Berry and Skett 1988). Since this effect was non-specific in nature, that is, both female- and male-specific enzymes were inhibited, elevations in cyclic AMP do not appear to be involved in mediating the feminising effects of GH, which is characterised by increased activity of the female-specific 5 α -reductase. A similar approach has precluded the role of protein kinase C in mediating the feminising effects of GH since phorbol esters, which mimic the action of endogenous diacylglycerol to activate protein kinase C, non-specifically inhibited androstenedione metabolism in isolated rat hepatocytes (Allan and Skett

(1988). However the role of PKC in GH action has been documented (Smal and De Meyts 1987).

It is, therefore, conceivable that the action of GH to decrease male-specific, cytochrome P-450-dependent 6 β -hydroxylase and 16 α -hydroxylase activities is a cyclic AMP- (PKA) or PKC-mediated event. Either PKA or PKC activation, which is capable of phosphorylation of cytochrome P-450 (Pyerin et al 1987) would result in reduced steroid-metabolising activity. In addition, GH was reported to suppress cytochrome P-450_{6 β} (gene IIIA1) activity (Yamazoe et al 1986) as well as other cytochrome P-450 isoenzymes (Waxman et al 1989b).

In conclusion, GH exhibited a significant feminisation of steroid metabolism in isolated male rat hepatocytes in the presence of both thyroxine and dexamethasone. Although the role of the permissive hormones, thyroxine and dexamethasone, in the manifestation of the feminising effects of GH is unclear, their involvement in this system has been widely documented (Colby 1980, Mode et al 1989, Guzelian et al 1988, Zaphiropoulos et al 1988, van der Hoeven and Guzelian 1987, Kriz et al 1982). The apparent requirement of these hormones in the culture medium in order to achieve feminisation of steroid metabolism strongly suggests that GH may be interacting with these hormones in vivo in order to produce its feminising effects. Thus treatment of isolated hepatocytes (and therefore removal of additional hormonal influences) with GH alone did not exhibit feminisation of steroid metabolism, whilst inclusion of dexamethasone and thyroxine in the culture medium, which

may serve to mimic the situation thought to exist in vivo, resulted in the feminisation of steroid metabolism. The mechanism of action by which GH feminises steroid metabolism is, as yet, unclear, although the phosphorylation of cytochrome P-450 may be involved in the inhibition of male-specific activities. However the method of inhibition or stimulation of the flavin-dependent enzymes is yet to be elucidated.

4.8 STUDIES WITH THE PUTATIVE INSULIN MEDIATOR

SUBSTANCE

Although the precise mechanism by which insulin elicits the vast array of its effects is not yet fully known, a unifying pathway of insulin action has been proposed recently (Brautigan and Kuplic 1988). Insulin, upon binding to its receptor α -subunit, activates the intrinsic tyrosine kinase residing in the transmembrane β -subunit. This activated kinase is thought to phosphorylate a G-protein, possibly G_{ins} (Larner 1988), which in turn activates a membrane PI-specific phospholipase C that is responsible for the generation of the putative mediators of insulin action from membrane-bound phosphatidylinositol-glycan precursors (Saltiel 1987). This is analagous to the insulin-induced release of heparan sulphate and alkaline phosphatase, both of which are also anchored to the plasma membrane by glycosyl-phosphatidylinositol glycan structures (Low and Saltiel 1988).

Numerous reports have accumulated over the past decade

following the initial proposition of the existence of low molecular weight mediators of insulin action by Lerner et al in 1979. The insulin mediators (polar head group, IP-glycan) are linked by a phosphodiester linkage to the parent phospholipid that is susceptible to hydrolysis by an insulin-sensitive PI-specific phospholipase C. The observation that the substance generated by treatment of purified membranes with either bacterial phospholipase C (Fox et al 1986, Kelly et al 1986) or phospholipase C purified from liver plasma membranes (Fox et al 1987) mimicked the effects of insulin reinforced the idea of a mediator of insulin action.

In the present study, insulin was reported to act as a general stimulatory agent of steroid metabolism (section 4.4), as has also been demonstrated by Hussin and Skett (1987), although the mechanism by which insulin acts has not yet been fully characterised. A number of reports have demonstrated that the isolated insulin mediator mimics the effects of insulin in most cases (Table 4). We therefore isolated this insulin mediator substance from rat hepatocytes treated with insulin, and determined the effects of this extract on hepatic steroid metabolism.

In order to test whether or not the insulin mediator generated in this study was active in a system used previously, we assayed the effects of incubation with the extract on mitochondrial pyruvate dehydrogenase activity (PDH) (Suzuki et al 1987). As is shown in Figure 47, insulin stimulated PDH activity in a dose-dependent manner,

exhibiting an optimum at physiological concentrations. Incubation of mitoplasts with various dilutions of the insulin mediator extract prepared in response to 10^{-9} M insulin (Figure 48) showed a concentration-dependent effect on PDH activity, and again an optimum concentration was apparent. Since the undiluted extract was less potent than the 1:100 dilution in stimulating PDH activity, a certain degree of amplification of the insulin signal was observed, in agreement with the findings of Newman et al (1985). This is consistent with the idea of hormone-stimulated activation of a second messenger system, where one molecule of insulin presumably causes the release of many molecules of the insulin mediator by a cascade mechanism. Thus the response observed with the undiluted extract may represent desensitisation due to the presence of excessive mediator. Alternatively, it has been reported that a stimulatory mediator is generated by low insulin concentrations, and an inhibitory mediator by high insulin concentrations (Saltiel et al 1982). However, the ethanol extraction step in the present study is likely to have removed the ethanol-soluble inhibitory insulin mediator from the extract used (Suzuki et al 1987). The effects of incubation with the insulin mediator on PDH activity are in agreement with recent reports (Jarett and Seals 1979, Saltiel et al 1981, Newman et al 1985), thus showing that the insulin mediator extract generated in our system is similar in action to that documented in the literature.

Incubation of hepatocytes with insulin mediator extracts prepared in response to various doses of insulin

showed that the insulin mediator stimulated steroid metabolism in a dose-dependent manner and exhibited maximum stimulation of enzyme activity at physiological concentrations (Figure 49). This is consistent with the dose-dependent effects observed with insulin previously (Figure 13), therefore showing that the insulin mediator is active and appears to mimic the stimulatory effects of insulin on steroid metabolism. Thus the insulin mediator substance may be mediating the effects of insulin in this system. A similar dose-response relationship has been obtained with the insulin mediator induced increase of cyclic GMP-activated cAMP phosphodiesterase (Pyne and Houslay 1988) and of PDH activity (Suzuki et al 1987).

The time course of generation of the insulin mediator was optimal after 5 minute exposure to insulin (Figures 50 & 51), which is consistent with previous reports (Pyne and Houslay 1988, Newman et al 1985, Suzuki et al 1987). In addition, optimal mediator generation at such an early time point is consistent with this insulin mediator substance being the second messenger of insulin action and agrees with the time course of insulin action observed earlier (Figure 15). Time course studies of incubation with the insulin mediator extract showed that steroid metabolism was stimulated in a similar manner to that with insulin (Figure 52) and enzyme activity was not significantly stimulated early in the time course, in accordance with the time course of generation of the insulin mediator substance (Figures 50 & 51).

Incubation of hepatocytes with various dilutions of the insulin mediator extract exhibited a concentration-dependent stimulation of enzyme activity (Figure 53), in agreement with the results obtained with PDH and glucose-6-phosphatase activities (Suzuki et al 1984) and lipid synthesis (Caro et al 1983). In addition, a dissociation of the effects of insulin on cytochrome P-450-dependent and independent enzymes was observed in that the latter were relatively unresponsive to increases in enzyme activity upon treatment with the insulin mediator extract (Figure 50). In contrast, no such differences have been reported upon incubation with insulin (Hussin and Skett 1987), and so this result may reflect different mechanisms of action for the stimulation of cytochrome P-450-dependent and independent enzymes by insulin.

The precursor phosphatidylinositol-glycan (PI-glycan) was reported to be located in the plasma membrane of rat liver, and relatively absent from subcellular organelles. Further, the PI-glycan appears to be localised specifically at the outer extracytoplasmic surface of the cell in both hepatocytes (Alvarez et al 1988) and adipocytes, where treatment of intact cells with PI-specific phospholipase C mimicked insulin action (Saltiel and Sorbara-Cazan 1987). This is in accordance with the general idea that proteins anchored to the cell membrane in the form of PI-glycans are located on the outer surface of the plasma membrane (Low and Saltiel 1988). If such a PI-glycan is the precursor of the insulin mediator, as has been proposed (Saltiel 1987), then

the insulin mediator must be generated outside the cell. This is supported by numerous reports that the insulin mediator is active when added to intact hepatocytes (Caro et al 1983) and adipocytes (Kelly et al 1988). Further, uptake of the insulin mediator into the cell rather than interaction with the insulin receptor appears to be involved (Caro et al 1983) since the insulin mediator mimics the effects of insulin in cell-free systems (Saltiel 1987). Since the insulin mediator affects intracellular target enzymes, a carrier system is required to transport the insulin mediator from the extracellular to the cytosolic face of the cell. In addition, passive diffusion of the polar head group is not likely due to the charged nature of the molecule. Although a recognition protein (receptor) for the IPG has not yet been identified, a mediator transport mechanism has been proposed by Romero et al (1988). In addition, the uptake, and therefore action, of the insulin mediator was blocked by inositol phosphate (Saltiel and Sorbara-Cazan 1987). Since the insulin mediator is also thought to contain an inositol phosphate moiety, the attenuation of the effects of the insulin mediator in the presence of inositol phosphate strongly suggests the blockade of its transport mechanism. In the present report, the observation that higher concentrations of the insulin mediator are required to mediate stimulation of steroid metabolism in intact hepatocytes as compared to the concentrations required for PDH activation, may represent the dependence of sufficient insulin mediator molecules being transported to the cell cytosol by this carrier

system.

Having established that the effects of insulin on hepatic steroid metabolism were mimicked by incubation with the insulin mediator extract, although to a lesser extent in the case of cytochrome P-450-independent enzymes (Figure 53), the striking interaction observed between insulin and GH (Figure 13) was further studied. As is illustrated in Figure 54, GH antagonised the stimulatory effects of the insulin mediator on steroid metabolism, which is reminiscent of the effects seen with insulin and GH (Figure 13). Thus the interaction observed between insulin and GH appears to take place distal to the step of insulin mediator generation from the plasma membrane since the same interaction was exhibited with the insulin mediator substance itself and GH (Figure 54).

A possible mechanism of action to account for this interaction could be the reported repression of certain cytochrome P-450 forms by GH (Yamazoe et al 1989), and so control may be exerted directly at the level of cytochrome P-450 either by phosphorylation-dephosphorylation (insulin), or by repression (GH). Alternatively, phosphorylation of cytochrome P-450 by either GH-activated PKA or PKC would also result in the inhibition of enzyme activity (Pyerin et al 1987).

The effects of the insulin mediator have been reported to mimic the actions of insulin in both intact cell systems and in membrane preparations (Cheng and Lerner 1985). Incubation of liver microsomes with various dilutions of the insulin mediator extract showed that diluted extracts were

more potent in stimulating steroid metabolism than the undiluted extract (Figure 55). This is similar to the concentration effects observed with mitochondrial PDH activation previously (Figure 48), and with other studies (Caro et al 1983, Suzuki et al 1987, Pyne and Houslay 1988), but does not correlate with identical studies carried out in intact hepatocytes (Figure 53). As was mentioned earlier, this discrepancy in insulin mediator potency between hepatocytes and microsomal preparations may reflect the requirement for a carrier process to transport insulin mediator molecules into the cell cytoplasm. Such a process would not be required with subcellular preparations (such as mitoplasts or microsomes), and so lower concentrations of the insulin mediator are effective as compared to the situation with hepatocytes.

A number of studies have described the purification of the insulin mediator extract (Saltiel 1987, Cheng and Lerner 1985, Romero et al 1988). In the present study, insulin mediator extracts were applied undiluted to AG1x8 ion-exchange columns and sequentially eluted with increasing concentrations of ammonium formate. Each of the fractions were then assayed in order to detect where the insulin mediator was eluted. As is shown in Figure 56, some stimulation of enzyme activity was observed with the 0.2M and 0.5M ammonium formate fractions, but it has been reported that nucleotides are primarily eluted at these concentrations (Suzuki et al 1987). Cytochrome P-450-dependent activities were markedly stimulated by the

contents of 1.0M ammonium formate fraction (fraction 1), whereas the cytochrome P-450-independent activities remained unresponsive until the next fraction. Significant increases in enzyme activity were also observed upon incubation with the 1.0M formic acid fraction, and it is thought this may represent insulin mediator not eluted by 1.0M ammonium formate. Merida et al (1988) have reported multiple phosphorylation states of the insulin mediator, and so the residual insulin mediator activity eluted by 1.0M formic acid in the present study may represent a different phosphorylated form to that eluted by 1.0M ammonium formate. In contrast to the elution profile of the insulin mediator reported in this study and by Suzuki et al (1987), Merida et al (1988) showed that insulin mediator generated upon treatment of purified rat hepatocyte glycopospholipid with *B. cereus* PI-specific phospholipase C, was eluted in three distinct peaks with 100mM, 200mM and 500mM ammonium formate upon Dowex AG1x8 chromatography. The 500mM fraction was the most potent in the inhibition of cyclic AMP-dependent protein kinase and represented a highly phosphorylated form of the insulin mediator. The authors therefore proposed that three forms of the insulin mediator with different phosphorylation states (2, 3, 4 phosphate groups) were present and that the extent of phosphorylation determined biological activity (Merida et al 1988). Incubation with various dilutions of fraction 2 of the AG1x8-purified insulin mediator exhibited dose-dependent stimulations of steroid metabolism in both microsomes (Figure 58) and hepatocytes (Figure 57). However this stimulation of enzyme

activity was more marked in the case of the microsomal preparation, again implying that transport of the insulin mediator into hepatocytes may be the hindering factor responsible for lower potency of the insulin mediator in this preparation. Co-incubation of microsomes with purified insulin mediator and GH resulted in decreases in enzyme activity (Figure 59), thus showing that the fraction 2 does indeed contain the insulin mediator since its effects were antagonised by GH.

Preliminary experiments to attempt to elucidate the nature of the components of this insulin mediator by paper chromatography and thin layer chromatography (Figure 60) showed that although carbohydrates such as galactose, mannose, glucosamine, and galactosamine, and inositol are present, more sensitive techniques are required to distinguish these compounds. The presence of these sugar components in the purified, acid-hydrolysed insulin mediator though, even at this level, shows homology to compounds reported by other authors. The constituents of the insulin mediator are thought to be inositol, glucosamine, galactose and phosphate (Saltiel 1987, Mato et al 1987b), although an insulin mediator containing mannose and galactosamine residues has recently been identified (Larner et al 1988). In fact, a whole family of different insulin mediators has been reported (reviewed by Cheng and Larner 1985, Gottschalk and Jarett 1985, Romero et al 1988). It has been postulated that the differences in the components of the insulin mediator may reflect either tissue differences or species

differences (Larner et al 1988). Conclusive proof of the involvement of the insulin mediator in insulin action will, however, be provided if structural analogues or synthetically prepared insulin mediators also mimic the actions of insulin.

A possible mechanism of action of the insulin mediator on the stimulation of steroid metabolism would undoubtedly involve a phosphorylation-dephosphorylation control mechanism (Avruch et al 1982, Larner et al 1979). Insulin is known to stimulate protein dephosphorylation by activating protein phosphatases as well as by promoting phosphorylation of other proteins by protein kinases (Espinal 1987). In addition, it has recently been suggested that insulin-induced phosphorylation reactions may be mediated by serine kinases (Espinal 1987), which may be activated by tyrosine kinases or directly by the insulin mediator itself (Czech et al 1988). Certainly, in the case of activation of PDH, it has been shown that activation of the PDH phosphatase, intrinsic to the PDH complex, and therefore α -subunit dephosphorylation, is responsible for the stimulation of PDH activity by the insulin mediator (Jarett et al 1980, Kiechle et al 1980, Suzuki et al 1984, Macaulay and Jarett 1985). In addition, the effect of insulin to increase glycogen synthase activity was also mimicked by the insulin mediator (Armstrong and Newman 1987). Protein phosphatase I, which is responsible for the dephosphorylation of glycogen synthase, glycogen phosphorylase, glycogen phosphorylase kinase, acetyl CoA

carboxylase and hormone sensitive lipase is activated by insulin (reviewed by Brautigan and Kuplic 1988) and this may constitute the mechanism by which the insulin mediator affects these enzymes.

The insulin mediator mimicked the phosphorylation-dephosphorylation effects of insulin on a number of target proteins in intact adipocytes (Alemany et al 1987). For instance, the phosphorylation of ATP citrate lyase was mimicked by the insulin mediator in a time- and dose-dependent manner, as was the dephosphorylation of hormone-sensitive lipase. In addition, both insulin and the insulin mediator blocked the isoprenaline-induced phosphorylation of glycogen phosphorylase and hormone sensitive lipase. Both insulin and the insulin mediator inhibited the phosphorylation of phospholipid methyltransferase in intact adipocytes (Kelly et al 1987), an enzyme that is regulated by a phosphorylation-dephosphorylation mechanism. The reduction in glucagon-stimulated glycogen phosphorylase a and pyruvate kinase activities by the insulin mediator was thought to occur by reducing the phosphorylation of these enzymes since cAMP-dependent protein kinase activity was also inhibited by the insulin mediator (Villalba et al 1988).

Both stimulation of PKA and PKC have been reported to result in significant decreases in steroid-metabolising activity (Berry and Skett 1988, Allan and Skett 1988), which correlates well with the inactivation of cytochrome P-450 upon phosphorylation by PKA or PKC (Pyerin et al 1987). In addition, a phosphorylation-dependent mechanism has been

proposed in the action of insulin on steroid metabolism (AH Hussin : personal communication). Thus if either insulin or the insulin mediator act by inhibition of protein kinase, this would result in reduced cytochrome P-450 phosphorylation and therefore increased steroid-metabolising activity. However, neither cAMP nor PI turnover appeared to be functional in insulin-stimulated steroid metabolism (AH Hussin : PhD thesis), and so this phosphorylation-dephosphorylation control may be exerted by serine/threonine phosphatases. Certainly, the insulin mediator has been reported to phosphorylate serine/threonine kinases (Czech 1988, Lerner 1988), and this may constitute the mechanism of insulin action rather than PKA or PKC activation. Contrary to these results, however, both insulin and the insulin mediator have been reported to alter the activities of various components of the cyclic AMP system. Insulin is known to antagonise hormone-stimulated cyclic AMP levels. In agreement with this, the insulin mediator did not alter basal cyclic AMP levels in hepatocytes, but reduced glucagon-stimulated cyclic AMP content (Alvarez et al 1987), possibly by its action to elevate low K_m phosphodiesterase activity (Kiechle and Jarett 1981, Saltiel et al 1986) or to decrease adenylate cyclase activity (Pyne and Houslay 1988). Insulin mediator generated from insulin-treated diabetic rats also inhibited the catalytic subunit of adenylate cyclase and cAMP-dependent protein kinase (Malchoff et al 1987) at a site distinct from the ATP-binding site (Villalba et al 1988). The effect of the insulin mediator on cAMP phosphodiesterase activity was

reported to be due to an increased phosphorylation of the enzyme (Marchmont and Houslay 1980). However insulin action to inhibit cAMP accumulation was demonstrated in the presence of phosphodiesterase inhibitors (Davidson and Berliner 1974), suggesting that adenylate cyclase inhibition was mediating the effects of insulin. The cAMP-lowering effect of insulin is, however, thought to be linked to phosphodiesterase inhibition in hepatocytes (Zhang et al 1983). Since both cAMP-dependent and cAMP-independent effects of insulin are mimicked by the insulin mediator, its generation is likely to be an early event following insulin binding to its receptor. The mechanism of action involved in the activation of the cytochrome P-450-independent enzymes remains obscure at this stage.

Diacylglycerol production upon hydrolysis of the PI-glycan (Saltiel et al 1987) may directly be involved in PKC activation. PKC has been reported to be involved in the action of insulin in BC3H-1 myocytes (del C. Vila et al 1989, Farese et al 1988), although this has been disputed (Spach et al 1986, Blackshear et al 1987, Chu et al 1987, Klip et al 1987).

A number of criteria for the involvement of insulin mediators in the action of insulin have been proposed (Armstrong and Newman 1987) :

- 1) The insulin mediator is generated by physiological concentrations of insulin.

-This criterion has been satisfied in the present study and in other reports (Suzuki et al 1987, Pyne and Houslay

1988). In addition, physiological concentrations of insulin appeared to generate optimal amounts of the insulin mediator.

2) The time course of the generation of the insulin mediator should be consistent with the onset of insulin action.

-The time course of insulin mediator generation correlated well with the stimulation of steroid metabolism both by insulin and the insulin mediator in this study. A similar result was obtained regarding cAMP phosphodiesterase activity (Pyne and Houslay 1988).

3) The insulin mediator substance must be generated by all insulin-responsive cell types.

-The insulin mediator has been generated from a number of systems upon exposure to insulin (Table 4). However, streptozotocin-diabetic rats failed to generate the insulin mediator upon treatment with insulin (Amatruda and Chang 1983). Similarly PDH activation was not achieved in response to insulin in adipocytes from NZO mice (Macaulay and Jarett 1988). G_i was reported to be abolished in diabetes (Gawler et al 1987) and this may result in impaired activation of the G-protein-activated phospholipase C, and thus the loss of insulin sensitivity in diabetic animals.

4) The insulin mediator should mimic the effects of insulin.

-The insulin-mimetic actions of the insulin mediator have been widely documented (Table 4), and insulin mediator mimicked the stimulatory effects of insulin on hepatic steroid metabolism in the present study.

5) The insulin mediator should be degraded by tissues at a rate consistent with insulin action.

-The duration of action of the insulin mediator to stimulate steroid metabolism, that is, loss of activity after 30 minutes of incubation, may reflect degradation of this substance at that time.

6) Chemically synthesised analogues of the insulin mediator should possess insulin-like activity.

-This approach will conclusively confirm the involvement of insulin mediators in insulin action.

This study therefore provides considerable evidence to support the role of the insulin mediator in insulin action.

In conclusion, the insulin mediator extract prepared upon treatment of hepatocytes with insulin mimics the stimulatory effects of insulin on steroid metabolism in a dose-dependent manner. The time course of both insulin mediator generation and action is consistent with the role of the insulin mediator as a second messenger of insulin action. Microsomal preparations appeared to be more responsive to the insulin mediator than intact hepatocytes in the stimulation of steroid metabolism, implying that transport of the insulin mediator into the cell is involved. In addition to mimicking the stimulatory effects of insulin, the effects of the insulin mediator were also antagonised by GH, thus indicating that interaction between the two hormones occurs distal to the step of insulin mediator generation. The insulin mediator extract purified by AG1x8 ion-exchange chromatography and eluted by 1.0M ammonium

formate also exhibited dose-dependent effects, although once again the microsomal system appeared to be more responsive than the intact hepatocytes. This effect was also antagonised by GH, showing that this fraction contained insulin mediator activity.

Paper chromatography of the purified, acid-hydrolysed insulin mediator substance showed the presence of carbohydrate components in the molecule, although exact identification of these requires a more sensitive technique.

The mechanism by which the insulin mediator acts probably involves a phosphorylation-dephosphorylation control at the level of cytochrome P-450, as does the interaction with GH. However, the mechanism of activation of the cytochrome P-450-independent activities remains unexplained.

4.9 GENERAL DISCUSSION

1) GH alone exhibited no significant effects upon administration to isolated rat hepatocytes. In contrast, GH is known to cause the feminisation of steroid metabolism in vivo (Mode et al 1981).

2) GH did not alter intracellular cyclic AMP content, and the effects of GH on steroid metabolism do not appear to be mediated by insulin-like growth factors, which exhibited an insulin-mimetic action in this system.

3) Insulin stimulated steroid metabolism in a non-specific manner, as has been reported previously (Hussin and Skett 1987), an effect that was strongly antagonised by GH, although in a sex-independent manner.

4) Dexamethasone stimulated steroid metabolism in a non-specific manner, and enzyme activity was restored to control levels upon incubation with GH in a dose-dependent manner.

5) Thyroxine exhibited concentration- and sex-dependent effects in short incubation studies. Co-incubation with GH resulted in marked decreases in enzyme activity in a sex-independent manner. Prolonged exposure to thyroxine in cultured hepatocytes exhibited no significant effects until 72h of incubation. Further treatment with GH restored enzyme activity to control values.

Thus GH appeared to non-specifically modulate the effects of insulin, dexamethasone and thyroxine on steroid metabolism in isolated hepatocytes.

6) Incubation of hepatocytes with GH in the presence of a combination of dexamethasone and thyroxine resulted in the feminisation of steroid metabolism, that is, an increase in female-specific and a decrease in male-specific activities. Thus dexamethasone and thyroxine appeared to play a permissive role in the action of GH to feminise steroid metabolism in isolated male rat hepatocytes. An extension of

this observation to the situation in vivo clearly indicates an interaction of GH with at least glucocorticoids and thyroid hormones in achieving its feminising effect.

7) The effects of insulin on hepatic steroid metabolism appear to take place by the generation of an insulin mediator substance. This insulin mediator was most active upon use of physiological concentrations of insulin, as measured by its ability to stimulate both PDH activity and hepatic steroid metabolism. The enhanced potency of the insulin mediator in microsomes and mitoplasts, as compared to the situation with hepatocytes was thought to be due to the involvement of a carrier mechanism present on the hepatocyte plasma membrane. In agreement with the results obtained with insulin, GH antagonised the stimulatory effects of the insulin mediator in a non-specific manner.

8) Insulin mediator activity was detected in the 1.0M ammonium formate and 1.0M formic acid eluates upon AG1x8 ion-exchange chromatography. Acid hydrolysis followed by paper chromatography of this AG1x8-purified insulin mediator extract showed that carbohydrate components were present, although their exact nature could not be determined due to the limitations of the technique used.

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